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UNIVERSITY OF ALBERTA

Characterization of the *cya-5* gene of *Neurospora crassa*

BY ©

Rajvir Dhillon

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

DEPARTMENT OF GENETICS

Edmonton, Alberta
Fall, 1993

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Rajvir Dhillon
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August 19, 1993

If I should pass the tomb of Jonah
I would stop there and sit for awhile;
Because I was swallowed one time deep in the dark
And came out alive after all

Carl Sandburg, Losers

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "CHARACTERIZATION OF THE *cya-5* GENE OF *Neurospora crassa*" submitted by Rajvir Dhillon in partial fulfillment for the degree of Master of Science.

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Date: Aug. 13/93

To Darshan and Harbans

Abstract

The *cya-5-34* nuclear mutant of *Neurospora crassa* was initially isolated as a slow-growing, tetrazolium non-reducing strain. Further analysis demonstrated that it was deficient in spectrally detectable cytochrome *aa3*, and accordingly lacked cytochrome oxidase activity. The absence of cytochrome oxidase activity was specifically attributed to the lack of subunit 1 (COX 1) of the enzyme. This was demonstrated by attempting to immunoprecipitate the subunits of the complex with an antibody raised against holocytochrome oxidase and with subunit specific antibodies.

In this study the deficiency in COX 1 of the *cya-5* mutant was confirmed by radioactively labelling mitochondrial translation products. The COX 1 protein was undetectable in mutant strains. The *cya-5*$^+$ gene was previously cloned by complementation of the mutant in transformations with a wild-type genomic library. The gene was sub-cloned into an approximately 6.4 kb SphI-XmaI fragment, the majority of which was sequenced. In this study, the approximately 1.5 kb that was remaining to be sequenced was completed. Partial cDNA clones obtained from a wild-type library and by the RACE procedure (Rapid Amplification of cDNA Ends) have identified the 3' terminus of the *cya-5*$^+$ transcript and 3536 bp of *cya-5*$^+$ cDNA sequence, but the 5' end of the gene has not been

characterized. Examination of the sequence obtained from the genomic and partial cDNA clones suggest that the *cya-5*$^+$ gene contains no introns, and that the first methionine codon of the protein coding reading frame is the *cya-5*$^+$ translation initiation site. Evidence for the latter comes from at least two observations. The sequence immediately preceeding this methionine codon displays similarity to the consensus sequence established for *N. crassa* translation initiation sites. In addition, sequence analysis predicts a mitochondrial targeting peptide, which are most often found at the amino terminus of proteins, immediately downstream of this methionine codon. Thus, the *CYA-5* protein is predicted to be 1136 amino acids long.

The *CYA-5* protein appears to be expressed at very low levels as suggested by codon usage analysis of the DNA sequence, and by Northern analysis, in which the *cya-5*$^+$ transcript was undetectable under conditions that allowed detection of the transcripts from the *cyt-2* gene, which is known to be expressed at low levels. Northern analysis of mitochondrial RNA from mutant and wild-type strains has revealed that the COX 1 mRNA is present at apparently wild-type levels in the *cya-5* mutant. This suggests that *CYA-5* affects COX 1 expression at the post-transcriptional level. This is supported by the finding that transcripts from the cytochrome *b* (COB) gene, which is transcribed from the same promoter as COX 1, are also present at normal levels in the *cya-5* mutant.

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Abbreviations

Amp^R	ampicillin resistance
ATP	adenosine triphosphate
bp	base pair
bisacrylamide	N, N' -methylenebisacrylamide
Ben^R	benomyl resistance
bromophenol blue	3', 3", 5', 5" tetrabromophenol-sulfonphthalein
BSA	bovine serum albumin
cDNA	complementary DNA
COB	cytochrome *b*
COX1	cytochrome oxidase subunit 1
COX2	cytochrome oxidase subunit 2
COX3	cytochrome oxidase subunit 3
cpm	counts per minute
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dITP	2'-deoxyinosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ddATP	2', 3'-dideoxyadenosine 5'-triphosphate
ddCTP	2', 3'-dideoxycytidine 5'-triphosphate
ddGTP	2', 3'-dideoxyguanosine 5'-triphosphate

ddTTP	2', 3'-dideoxythymidine 5'-triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl-ß-D-thiogalactoside
kbp	kilobase pairs
mCi	milliCurie
MMLV	Moloney murine leukemia virus
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	messenger RNA
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
N. crassa	*Neurospora crassa*
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
RACE	Rapid Amplification of cDNA Ends
RNA	ribonucleic acid
rpm	revolutions per minute
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisiae*
S. pombe	*Schizosaccharomyces pombe*
SDS	sodium dodecyl sulfate

Tet^{R}	tetracycline resistance
Tris	tris (hydroxymethyl) aminomethane
Triton X-100	octylphenoxypolyethoxyethanol
tRNA	transfer ribonucleic acid
URF	unidentified reading frame
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

Introduction

Mitochondria perform several essential functions in the eukaryotic cell, including those involved in cellular respiration. The vast majority of proteins present in mitochondria are encoded by the nuclear genome. These proteins are translated on cytosolic ribosomes and imported into mitochondria (Hartl et al., 1989). However, a small number of proteins, that is, several subunits of enzymes involved in oxidative phosphorylation, are encoded by the mitochondrial genome. These proteins are translated on mitochondrial ribosomes (Tzagoloff, 1982).

We owe our current knowledge of the role of nuclear and mitochondrial genes in mitochondrial biogenesis to the early work that established mitochondrial genetics. The first cytoplasmic mutants characterized were in *Saccharomyces cerevisiae* (Ephrussi and Hottinguer, 1951). These mutants gave rise to small colonies due to a respiratory deficiency, and were thus named petite mutants. They arose at a high frequency spontaneously, and their frequency could be greatly induced by the mutagen acriflavine. When mated to normal cells, they gave rise to normal diploids; upon sporulation, asci were produced in which all spores were normal. Repeated backcrosses to the mutant did not recover the mutant phenotype. The observed non-Mendelian inheritance of the mutant character was ascribed to the loss or inactivation of a cytoplasmic, autoreproducing factor required for the normal synthesis of respiratory enzymes (Ephrussi and Hottinguer, 1951). A short time afterwards, a slow-growing, respiratory deficient mutant that also showed non-Mendelian behaviour in crosses was identified in *Neurospora crassa* (Mitchell and Mitchell, 1952). The mutant, named poky, was characterized as maternally inherited, only transmitted when the mutation was carried by the protoperithecial parent. It was suggested that the poky mutation was due to a cytoplasmic factor. The discovery of DNA in mitochondria of yeast (Schatz et al., 1964) and *Neurospora* (Luck and Reich, 1964), and the demonstration in yeast petite mutants of alterations in mitochondrial DNA (Mounolou et al., 1966), suggested that cytoplasmically inherited characteristics were encoded by mitochondrial DNA (mtDNA). Soon after the first cytoplasmic mutants were characterized, nuclear respiratory deficient mutants were identified in yeast and *N. crassa* (Chen et al., 1950; Mitchell et al., 1953). These were the first nuclear mutants identified that were defective in genes involved in mitochondrial function. These studies layed the groundwork for future studies that determined the nature of the mitochondrial genome and its functions.

The mitochondrial genome and its expression

The basic function of the mitochondrial genome in all eukaryotes is to encode components of its own translation machinery (i.e. rRNAs and tRNAs) and certain proteins that function in electron transport and oxidative phosphorylation in mitochondria. However, the number and type of genes encoded by mitochondrial genomes varies greatly among plants, animals, fungi and protists (Gray, 1989). Only a few genes appear to be common to all characterized mtDNAs. They are genes for the large and small subunit rRNAs, cytochrome oxidase subunit 1 (COX 1), and cytochrome b (COB). There is also a great deal of heterogeneity in the size and structure of mtDNA within and between plants, animals, fungi and protists.

Among animals alone, mtDNA is relatively conserved in gene content and structure. Mitochondrial DNA in vertebrates, in particular, show very little variation between species (Gray, 1989; Wolstenholme, 1992). The size of mtDNA in vertebrates ranges from 16-18 kb. The order of the genes among human, mouse, bovine and *Xenopus laevis* is invariant, and the genomes are extremely economical in organization. Virtually the entire vertebrate mitochondrial genome is comprised of coding sequence. There are no introns and little or no intergenic space (Attardi, 1985). The little size variation that is observed is confined to a 1-2 kbp non-coding region (the D-loop region), which contains promoters for transcription and origins or replication (Gray, 1989; Wolstenholme, 1992). Greater variation in size and structure of mtDNA has been observed among invertebrates (Wolstenholme, 1992). Size ranges from 14 kbp in the nematode worms *Ascaris suum* and *Caenorhabditis elegans* (Okimoto et al., 1992) to approximately 40 kbp in the sea scallop, *Plactopecten megellanicus* (Snyder et al., 1987). However, as in vertebrates, the size variation is primarily due to localized duplications of sequences in the non-coding control regions of mtDNA containing promoters for transcription and origins of replication (Wolstenholme, 1992). There are little or no non-coding sequences between genes. Thus, economy of organization of coding sequences is a common feature of all animal mitochondrial genomes. All animal mitochondrial genomes encode the same number and types of genes with few exceptions. They encode a complete set of tRNAs, the large and small subunit rRNAs, and 13 proteins. The proteins encoded by animal mitochondrial genomes are 7 subunits of complex 1 of the electron transport chain (NADH dehydrogenase), cytochrome *b* of coenzyme QH2-cytochrome *b* oxidoreductase (complex 3), 3 subunits (COX 1, COX 2 and COX 3) of cytochrome oxidase (complex 4), and 2 subunits of the ATP synthase complex (ATP 6 and 8). However, there is little conservation of gene order between different animal phyla (eg. vertebrates, insects, echinoderms and nematodes) (Gray, 1989; Wolstenholme, 1992).

There is much greater variation in mtDNA size, structure and gene content in fungi, plants and protists than observed in animals. In contrast to the economical organization of animal mitochondrial genomes, mtDNA in fungi, plants and protists largely consist of non-coding sequences between genes. Fungal mitochondrial genome sizes range from approximately 18 kbp in *Schizosaccharomyces pombe* to over 100 kbp (Wolf and Del Giudice, 1988; Clark-Walker, 1992). The size differences are chiefly the result of intergenic spacer lengths, the presence or absence of optional introns, and the presence and number of repetitive sequences (Wolf and Del Giudice, 1988; Gray, 1989; Clark-Walker, 1992). However, there are some instances of variation in the types of genes encoded. For example, *N. crassa* and *S. cerevisiae* mtDNAs each contain a gene for a ribosomal protein (Butow et al., 1982; LaPolla and Lambowitz, 1981). In addition, some introns in the COB and COX 1 genes of *S. cerevisiae* code for proteins, called maturases, responsible for intron splicing (Tzagoloff and Myers, 1986; Attardi and Schatz, 1988; Perlman and Butow, 1989). Intron encoded maturases also appear to be present in mitochondrial genes of *N. crassa, Aspergillus nidulans*, and other fungi (Brown, 1987; Burke et al., 1984). Several yeast mitochondrial genomes *(S. cerevisiae, S. pombe* and *Candida glabrata)* are devoid of genes for subunits of NADH dehydrogenase (complex 1), since mitochondria in these species do not contain a NADH dehydrogenase of the complex 1 type (Grivell, 1989; Clark-Walker, 1992). There is little conservation of mitochondrial gene order between species of fungi, suggesting that extensive rearrangements of mtDNA have occurred during evolution (Clark-Walker, 1992).

Plant mtDNA is the largest and most complex of eukaryotic mtDNAs. There is great heterogeneity in size and structure of mtDNA among plant species. Size varies over a 10-fold range, from 200 kbp to over 2400 kbp (eg. *Cucumis melo*), and there is little conservation of gene order (Gray, 1989; Hanson and Folkerts, 1992). The physical heterogeneity is largely due to directly repeated sequences which promote recombination, generating subgenomic circular molecules (Lonsdale et al., 1988; Hanson and Folkerts, 1992). Plant mitochondrial genomes have been shown to contain genes not detected in other mitochondrial genomes. For example, 5S rRNA genes have been detected in many plant species (Gray and Spencer, 1981; Chao et al., 1983; Hanson and Folkerts, 1992). Genes for mitochondrial ribosomal proteins (eg. rps12, rps13, rps14, and rps19) homologous to *E. coli* ribosomal proteins have been detected in several species (Hanson and Folkerts, 1992). Sequences from other genomes (i.e. the nucleus and chloroplast), particularily tRNA genes, have also been widely detected in plant mtDNA (Gray, 1989; Hanson and Folkerts, 1992).

Although protistan mtDNA has not been widely characterized, an astonishing amount of variation in information content, size and structure has been discovered. The smallest protist mitochondrial genome is that of *Chlamydomonas reinhardtii*, which is 15.8 kbp long, while that of *Tetrahymena* is as long as 55 kbp (Cummings, 1992). Linear mitochondrial genomes have been found in *Tetrahymena pyriformis* (Suyama et al., 1985; Cummings, 1992) and *Paramecium aurelia* (Pritchard et al., 1986; Cummings, 1992). Mitochondrial DNA from trypanosomatids is complex. It is composed of large maxicircles with interwound minicircles concatenated in special structures called kinetoplasts (Stuart and Feagin, 1992). Many of the genes found in mtDNA of animals are missing in various protistan mitochondrial genomes. *Chlamydomonas reinhardtii* mtDNA does not contain any genes for the ATP synthase complex, and is missing the common genes for the COX 2 and COX 3 subunits of cytochrome oxidase as well as most tRNA genes (Attardi and Schatz, 1988; Cummings, 1992). The kinetoplasts of Trypanosomatids lack genes for the ATP synthase complex and any tRNA genes (Gray, 1989; Stuart and Feagin, 1992). Only a small number of tRNA genes have been found in *Paramecium aurelia* and *Tetrahymena pyriformis* indicating that most mitochondrial tRNAs are encoded in the nucleus (Cummings, 1992).

The large degree of heterogeneity in the organization of the mitochondrial genome is accompanied by essential differences in the control of gene expression among various species. Sequences important for mitochondrial gene expression have been well studied in *S. cerevisiae*, *N. crassa* and human mtDNA. Despite the differences, a common feature of mitochondrial gene expression in these organisms is the occurrence of primary transcripts that include the information for more than one gene. Processing of primary transcripts is necessary to produce mature mRNAs, rRNAs and tRNAs.

In *S. cerevisiae* promoters for transcription were first identified by *in vitro* capping with Vaccinia virus guanylyltransferase (Christianson and Rabinowitz, 1983; Levens et al., 1981). These experiments identified a nonanucleotide sequence, 5'-ATATAAGTA-3' (with the last A being the transcription start site), which functions as a promoter. This was confirmed by *in vitro* transcription (Edwards et al., 1982; Biswas et al., 1987; Schinkel et al., 1987a). Other sequences that influence promoter strength have also been found (Biswas et al., 1987; Schinkel et al., 1987a). Contrary to what is frequently observed in *N. crassa* and human mitochondria, processing 5' and 3' to tRNA genes does not generate mature transcripts in yeast. Further processing is often necessary (Grivell, 1989; Breitenberger et al., 1985). Processing of primary transcripts appears to involve several endonucleases. An RNase P-like tRNA processing enzyme is responsible for cleaving primary transcripts on the 5' side of tRNAs (Grivell, 1989). The RNA component of this enzyme is encoded in mtDNA. A dodecamer sequence, AAUAAUAUUCUU, on the 3' end of mRNAs serves as a processing site to generate mature mRNA 3' termini (Osinga et al., 1984; Thalenfeld et al., 1983). Other endonucleases may be required for further processing of transcripts (eg. at the 5' end of mRNAs; Tzagoloff and Myers, 1986; Kubelik et al., 1990). Intergenic spaces in yeast mtDNA are highly AT rich interspersed with GC rich clusters (Bernardi, 1982), whose function is not understood, but have been shown to be mobile elements (Clark-Walker, 1992).

As mentioned above, the human mitochondrial genome as in other vertebrates, is extremely economical in organization, with virtually no intergenic spaces, no introns and no untranslated leader sequences (Anderson et al., 1981; Tzagoloff and Myers, 1986). The rRNA genes and most protein coding genes are flanked by tRNA genes, which are dispersed throughout the genome. The 5' and 3' ends of tRNA genes appear to act as signals for processing of primary transcripts from both strands of the genome (Ojala et al., 1981; Clayton, 1992), thus generating mature transcripts. Each strand is transcribed from a single promoter located in the D-loop region leading to primary transcripts that contain all the genes on each strand (Chang and Clayton, 1984; Clayton, 1991; 1992). Promoters for the heavy strand (HSP) and light strand (LSP) are separated by 150 bp and have a similar 8 bp core sequence 5' CC(G)CCAAAA(+4) 3' that includes the transcription initiation site. This supports a minimal amount of transcription. Additional upstream sequences are required for efficient transcription (Bogenhagen et al., 1984; Chang and Clayton, 1984; Hixson and Clayton, 1985; Clayton, 1991; 1992). A mitochondrial transcription factor, mtTF1, has been recently identified that binds upstream of transcription initiation sites to activate transcription (Clayton, 1992).

Neurospora crassa mtDNA, which ranges in size from 60 to 73 kb (Collins and Lambowitz, 1983), differs from yeast mtDNA in that it has fewer promoters. Longer transcripts, therefore, are often observed that contain the information for more genes (Green et al., 1981; Burger et al., 1985; Breitenberger et al., 1985). Using an *in vitro* transcription assay, at least 5 transcription initiation sites have been identified in *N. crassa* mtDNA (Kennell and Lambowitz, 1989; Kubelik et al., 1990). Sequences of these sites have defined an 11-nucleotide consensus sequence TTAG(A/T)RR(G/T)(G/C)N(A/T) as part of the *Neurospora* mtDNA promoter. Transcription initiation occurs at the fifth or sixth nucleotide of the consensus sequence. Promoters have been identified upstream of the small rRNA, large rRNA and cytochrome b genes. Active promoters are preceeded by AT rich regions (Kubelik et al., 1990).

Mature rRNA, tRNA and mRNAs are produced by processing of primary transcripts, presumably by endonucleases that recognize secondary structures generated upstream and downstream of each gene. In several cases, the 5' and 3' ends of tRNAs that flank genes are processing sites to generate mature mRNAs and rRNAs (Akins and Lambowitz, 1984; Breitenberger et al., 1985; Burger et al., 1985; de Vries et al., 1985). However, there are also protein coding genes that are not flanked by tRNA genes, and primary transcripts in these regions of the genome are processed at sites around secondary structures in the mRNA that substitute for tRNA sequences. This has been demonstrated for the region of mtDNA consisting of the ATPase subunit 6, cytochrome oxidase subunit 2, and the mitochondrial ATPase proteolipid-like genes, which is transcribed as one unit (Agsteribbe and Hartog, 1987).

Most of the genes of *N. crassa* mtDNA are flanked by highly conserved GC-rich palindromic sequences. Coincidentally, these sequences consist of two tandemly repeated PstI sites preceeded by a cluster of C's and followed by a cluster of G's. PstI palindromes, as they are commonly called, were proposed to serve as processing sites of primary transcripts to generate mature RNAs (Yin et al, 1981). However, this was shown not to be the case, since virtually every identified processing site is not associated with these sequences. There is good evidence that these GC rich clusters are mobile genetic elements (Clark-Walker, 1992).

Cytochrome c Oxidase

Cytochrome *c* oxidase is an enzyme of the mitochondrial respiratory chain. The enzyme catalyzes the oxidation of ferrocytochrome *c* and the reduction of molecular oxygen to water. The energy from this reaction is conserved as a pH gradient and membrane potential across the inner membrane, generated by H^+ consumption in the reaction, and by proton translocation through the inner membrane to the intermembrane space (Capaldi, 1990; Denis, 1986). The mammalian cytochrome *c* oxidase is comprised of 13 subunits, whereas only 12 and 7 subunits have been identified in *Saccharomyces cerevisiae* and *Neurospora crassa*, respectively (Capaldi, 1990; Denis, 1986; Power et al., 1984; Taanman and Capaldi, 1992). The bacterial enzyme is very simple in comparison, with only 3 subunits identified in most species examined. The three largest subunits of the eukaryotic enzyme, 1, 2 and 3, are encoded by the mitochondrial genome, whereas the remaining subunits are nuclear-coded. The enzyme is associated with 4 metal prosthetic groups that make up the redox centres of the complex: hemes a and a_3, CuA and CuB. There is a considerable amount of evidence that localizes the prosthetic groups to subunits 1 and 2 of the complex (Capaldi, 1990).

The *in vivo* membrane associated cytochrome *c* oxidase exists in the form of a dimer (Capaldi, 1990). The cytochrome *c* binding site is in a cleft on the cytoplasmic side of the inner membrane. The binding site has been localized primarily to subunit 2, but there may also be contributions of nuclear-coded subunits to cytochrome *c* binding (Capaldi, 1990).

Evidence that cytochrome *c* oxidase acts as a redox linked proton pump was initially provided by Wikstrom, who showed that oxidation of ferrocytochrome *c* by oxygen in rat liver mitochondria was associated with acidification of the external medium (Wikstrom, 1977). Studies since have suggested that the reaction consumes approximately 2 H^+/ferrocytochrome *c* oxidized (1 electron); one of these H^+ is used towards the formation of water, and 1 H^+ is translocated across the inner membrane to the intermembrane space (Brunori et al., 1987). The mechanism of proton pumping by cytochrome *c* oxidase is not well understood. Studies have shown that subunit 3 is primarily responsible for this function (Casey et al., 1980), although other subunits may also be involved (Capaldi, 1990). Several hypothesis have been proposed to explain how cytochrome *c* oxidase couples electron transport to proton translocation. Mitchell *et al.* (1985) have proposed an O-loop scheme similar to the Q-loop mechanism proposed for coupling in complex 3 (Mitchell, 1976). Alternatively, conformational coupling models have been proposed, which suggest that electron transfer is coupled to proton translocation by changes in protein conformation (Malstrom and Nilsson, 1988).

The mitochondrially encoded subunits 1, 2 and 3 comprise the catalytic core of the eukaryotic cytochrome *c* oxidase. Evidence suggests that this is the minimal unit competent for electron transfer and proton pumping functions. Bacterial oxidases, which consist of homologues of only these three subunits, are capable of both functions. Hence, the question remains as to the role of the subunits coded by the nucleus. It is generally believed that these subunits are involved in regulation of cytochrome oxidase function at the level of subunit assembly and/or catalytic function (Kadenbach and Merle, 1981; Dowhan et al., 1985; Capaldi, 1990).

Nuclear genes required for mitochondrial gene expression

Saccharomyces cerevisiae

Mitochondria are highly dependent on the nuclear genome, since the majority of mitochondrial proteins are nuclearly encoded. The reliance on nuclear gene products is clearly reflected in the fact that even proteins required for expression of the few mitochondrial genes, are predominantly coded in the nucleus. The assembly of functional mitochondria, therefore, involves the coordinated expression of both the nuclear and mitochondrial genomes. The mitochondrial genome carries some genes required for its own expression. These are components of the translation system, including tRNA genes, the small and large subunit rRNA genes, and in some instances, a single ribosomal protein (Tzagoloff and Myers, 1987). However, the genes for numerous other proteins required for mitochondrial gene expression, such as, RNA polymerase, ribosomal proteins, RNA processing endonucleases, and translation factors are found in the nucleus (Tzagoloff and Dieckmann, 1990). Many of these genes have been identified in the yeast *S. cerevisiae*, primarily through the analysis of mutants with respiratory defects (Tzagoloff and Dieckmann, 1990). Nuclear mutants in yeast which are respiration deficient are commonly referred to as nuclear petite or pet mutants, to distinguish them from the well characterized cytoplasmic petite mutants, which were first identified by Ephrussi and colleagues (see above).

Several laboratories over the last several years have used various approaches to isolate nuclear respiratory defective mutants of yeast (Tzagoloff and Dieckmann, 1990). The basic method of selection for respiratory mutants is based on their ability to grow on media containing a fermentable carbon source, such as glucose, but inability to grow on a non-fermentable carbon source, such as glycerol or lactate. These strains are also recognized by their colony morphology after plating of a mutagenized stock on medium containing a high concentration of glycerol with limiting amounts of glucose (0.1 to 0.2%). Both nuclear and cytoplasmic petite mutants form small colonies (as their name implies) on this medium, because they are unable to grow after the glucose in the medium is used up. Wild-type strains continue to grow after the glucose is depleted, and form larger colonies using glycerol as a carbon source (Tzagoloff and Dieckmann, 1990). To distinguish the nuclear mutants from the more abundant class of cytoplasmic petites, the respiratory deficient strains are then crossed to yeast tester strains that completely lack mtDNA (rho^o), and the diploid progeny are checked for growth on a non-fermentable substrate. Growth of the diploid implies that the defect is in a nuclear gene and is recessive. However, if the mutant is not complemented by the rho^o tester, the mutation is presumed to be in mtDNA.

Further analysis of PET genes represented in mutant collections has identified very few nuclear genes directly involved in the control of mitchondrial gene expression at the level of transcription. A mitochondrial RNA polymerase has been identified (Schinkel and Tabak, 1989). The RNA polymerase is composed of a 145 kDa core subunit encoded by the *RPO41* gene (Greenleaf et al., 1986), and at least one specificity factor, *MTF1* (Schinkel et al., 1987b; Jang and Jaehning, 1991). The *RPO41* gene product has non-specific transcriptional activity, and requires *MTF1* for promoter recognition (Schinkel et al., 1987b; Schinkel et al., 1988). Another mitochondrial transcription factor, *MTF2*, has also been identified (Lisowsky, 1990). Temperature sensitive collections of pet mutants that specifically lose or cause a decrease in mitochondrial mRNA levels have been sought (Mueller et al., 1987), which, in the future, may identify other RNA polymerase specificity factors or transcription factors.

The majority of characterized nuclear genes that affect mitochondrial gene expression in yeast act at the post-transcriptional level. Thus, nuclear mutants that are defective in mitochondrial mRNA processing, translation or post-translational events have been identified (Fox, 1986; Costanzo and Fox, 1990). Such mutants fall into two broad categories: those that are required for the expression of all or several mitochondrial genes, and those that control the expression of a specific gene.

The pet mutants of mitochondrial RNA processing fall into both categories. Proteins involved in RNA processing can function in splicing or maintaining stability of transcripts. An example of the latter is the *cbp1* gene product. This protein is required for the stability of the cytochrome *b* (COB) transcript (Dieckmann et al., 1984; Dieckmann and Mittelmeier, 1987). The COB gene is transcribed as a precursor containing the $tRNA^{Glu}$ 1100 bases upstream of COB coding sequences (Christianson et al., 1983). The precursor is processed to yield mature $tRNA^{Glu}$ and COB mRNA with a 954 base leader sequence. Mutants of *CBP1* have greatly reduced (but detectable) levels of COB mRNA, but transcription of COB appears not to be affected, since levels of $tRNA^{Glu}$ are normal (Dieckmann et al., 1984). Moreover, the *CBP1* protein has been mitochondrially localized (Weber and Dieckmann, 1990). The mutant phenotype suggests that *CBP1* plays no role in transcription, or in processing of $tRNA^{Glu}$. It appears, instead, that it is required to protect COB mRNA from degradation, or to generate a stable mRNA from an unstable precursor (Dieckmann et al., 1984; Dieckmann and Mittelmeier, 1987).

The *CBP1* protein acts on the 5' third of the untranslated leader of COB mRNA. This was demonstrated by isolating mitochondrial revertants of *cbp1* mutants, that are similar to revertants of mutations in several mRNA specific translation factors (see below).

The revertants encode chimeric COB transcripts, in which the COB leader sequence is replaced by the leader of another mitochondrial gene (Dieckmann et al., 1984; Dieckmann and Mittelmeier, 1987).

Several PET genes that function in splicing of mitochondrially encoded COB and COX 1 genes have been identified. The number of introns carried by these genes differs among strains of *S. cerevisiae*, with up to 8 introns in COX 1 and 5 introns in COB of KL14-4A, the so called "long" strain (Dujon, 1981). The excision of some introns of COX 1 and COB are dependent not only on nuclear encoded factors, but also on proteins encoded within the introns themselves (Lazowska et al., 1980; De La Salle et al., 1982; Weiss-Brummer et al., 1982; Lazowska et al., 1989; Perlman and Butow, 1989). The requirement for both nuclear and mitochondrial intron encoded proteins (called maturases) for splicing at least some group 1 and group 2 introns in COB and COX 1 was unexpected, for it was demonstrated that many of these introns (both group 1 and group 2 alike) are self-splicing *in vitro* (Garriga and Lambowitz, 1984; van der Horst and Tabak, 1985; Gampel and Cech, 1991). It has, therefore, been proposed that the function of the nuclear and mitochondrial encoded splicing factors *in vivo* is to facilitate and stabilize the correct folding of introns to allow splicing to occur. The splicing mechanism itself, however, is RNA (self) catalyzed (Gampel et al., 1989; Cech, 1990).

Analysis of many PET genes involved in mitochondrial RNA splicing has demonstrated that most of them have additional roles in mitochondrial gene expression (Grivell, 1989). All of these genes appear to function in translation, but there may be other functions as well. Since splicing of many mitochondrial introns requires translation of maturases encoded within them, it is difficult to decide whether many pet mutants have primary defects in splicing, or that their splicing defects are secondary effects of the inability to translate mRNAs. Nevertheless, evidence suggests that several proteins have primary functions in both splicing and translation of RNAs (Asher et al., 1989).

Among the proteins which perform functions in addition to RNA splicing are the products of several *NAM* (Nuclear Accomodation of Mitochondria) genes, mutants of which were isolated by Dujardin et al. (1980). The *nam* mutants were selected as suppressors of various mutations in mitochondrial DNA (mit- mutants). Mutations in *nam1, 2, 7* and *8* suppressed mitochondrial intron mutations, and were, therefore, classified as mitochondrial splicing factors. *Nam1, 7* and *8* mutants suppressed defects in various introns of COB and COX 1, whereas *nam2* mutants only suppressed defects in the fourth intron of the COB gene (bI4). Further analysis of the *nam* mutants, however, has revealed defects in mitochondrial translation, and perhaps other functions as well. Based on the phenotype of naml mutants, this gene product has been implicated in both translation and splicing of COX 1 mRNA (Asher et al., 1989). The *NAM7* protein, however, is required for splicing introns of both COX 1 and COB transcripts, as it suppresses several group 1 and 2 intron mutations in both genes (Dujardin, 1980). Evidence that *NAM7* is responsible for other functions in the cell comes from the observation that yeast strains devoid of mitochondrial introns remain respiratory deficient in a *nam7* mutant background (Altamura et al., 1992). Sequence analysis has revealed homology to 5 conserved motifs of the superfamily of replicative proteins with helicase activity (Hodgman, 1988), which has led Altamura *et al.* (1992) to propose a role for *NAM7* in translation.

The best evidence for a dual role in transcription and translation has been provided for the *nam2* gene product. *NAM2* is involved in splicing of the fourth introns of the COB (bI4) and COX 1 (aI4) genes, and has also been identified as a mitochondrial leucyl-tRNA synthetase (Dujardin et al., 1980; Groudinsky et al., 1981; Labouesse et al., 1985; Herbert et al., 1988). Compelling evidence for *NAM2* being directly required for splicing was provided by Labouesse (1990). The bI4 maturase was synthesized from a plasmid in the cytosol and imported into mitochondria in a *nam2* mutant. The maturase did not restore excision of the mitochondrial bI4 and aI4 introns (which is its normal function in wild-type cells). This implies that the splicing deficiency in *nam2* mutants is not simply a secondary effect of absence of the bI4 maturase due to a defect in protein synthesis. Interestingly, the mechanism of action of the *nam2* suppressor on bI4 mutations is by activation of the latent aI4 maturase, which, in turn, excises both aI4 and bI4 (Labouesse et al., 1985). In wild-type cells, bI4 maturase, in conjunction with the *NAM2* gene product, is responsible for excision of aI4, as well as its own (bI4) intron (De la Salle et al., 1982; Labouesse et al., 1984).

Other nuclear gene products required for mitochondrial RNA splicing and translation include the *MSS116* (Seraphin et al., 1989), *MSS51* (Simon and Faye, 1984; Decoster et al., 1990) and the *PET54* (Valencik et al., 1989; Valencik and McEwen, 1991) proteins. Interestingly, Valencik and McEwen (1991) were able to show that the translation and splicing functions of *PET54* are genetically separable, such that mutants defective in only one or the other of the two functions could be obtained.

The many examples of nuclear gene products with dual functions in splicing and translation implies that this is a general phenomenon in yeast, and may be prevalent in mitochondrial systems of other organisms as well (eg. the *cyt-18* gene product of *N. crassa*, see below). A consequence of this form of expression might be the coordination of synthesis of mitochondrial transcripts and their proteins. The advantages of this, if any, are unclear.

Some PET genes function solely in splicing. The most well studied of these genes, *cbp2*, codes for a protein responsible for excision of the terminal intron of the COB gene (McGraw and Tzagoloff, 1983). A mtDNA mutation that suppresses the *cbp2* defect is a precise excision of the last intron (bI2) from the COB gene (Hill et al., 1985). The revertants are otherwise completely normal, indicating that *CBP2* has no other function in the cell. The COB terminal intron can self-splice *in vitro* at Mg^{2+} concentrations 10-fold higher than physiological concentrations (eg. 50mM; Gampel et al., 1989). However, at physiological concentrations of Mg^{2+} (5mM) splicing of the bI2 intron is strictly dependent on the *CBP2* protein (Gampel et al., 1989). Thus, *CBP2* does not have a catalytic function, per se, in the splicing reaction, but stabilizes secondary structures required for the autocatalytic reaction to occur. Presumably, the correct secondary structures are favoured in high Mg^{2+} concentrations *in vitro*, even in the absence of proteins (Gampel et al., 1989; Gampel and Cech, 1991).

Nuclear control of mitochondrial gene expression in yeast occurs primarily at the level of translation (Falcone et al., 1983; Fox, 1986). The nucleus encodes many components of the general mitochondrial translation system, such as ribosomal proteins and tRNA synthetases (Costanzo and Fox, 1990). Analysis has had to rely primarily on genetics. Only a minority of the approximately 70 yeast mitochondrial ribosomal proteins (Mieszczak et al., 1988; Boguta et al., 1992) have been identified and examined. These include *MRP1* and *2* (Myers et al., 1987), *MRP3* and *4* (Tzagoloff and Dieckmann, 1990), *MRP13* (Partaledis and Mason, 1988), *PET123* (Haffter et al., 1990; McMullin et al., 1990), *YmL31* (Grohmann et al., 1989), *YMR-31* and *YMR-44* (Matsushita et al., 1989), MRP 20 and 49 (Fearon and Mason, 1992), *MRP7* (Fearon and Mason, 1988) and *NAM9* (Boguta et al., 1992). All of these ribosomal proteins are distinct from the cytosolic ribosomal components. In contrast, two genes for mitochondrial tRNA synthetases also specify the cytosolic forms of the enzymes. They are the histidinyl-tRNA synthetases (Natsoulis et al., 1986) and the valyl-tRNA synthetases (Chatton et al., 1988). In both cases, the mitochondrial and cytosolic forms are transcribed from alternate promoters. The mitochondrial form is transcribed from an upstream promoter that produces transcripts that code for N-terminal mitochondrial targeting signals. The other mitochondrial tRNA synthetases that have been identified are encoded by genes distinct from the genes encoding their cytosolic counterparts, as mutations in these genes produce a non-respiratory, but viable phenotype (Costanzo and Fox, 1990).

A number of nuclear genes have been identified that code for translational activators of specific mRNAs, rather than components of the general translation machinery. Nuclear activation of at least two mitochondrial proteins, COX 3 and COB, appears astonishingly complex. Three specific translational activators have been identified for each protein. Recessive mutations in nuclear genes *pet494, pet54* and *pet122* block accumulation of the COX 3 protein leading to a respiratory deficiency (Ebner et al., 1973; Cabral and Schatz, 1978; Muller et al., 1984; Costanzo et al., 1986; Kloeckener-Gruissem et al., 1988). The proteins act at the post-transcriptional level, since COX 3 mRNA is present at wild-type levels in each of the mutant strains. Mitochondrial suppressors of all three nuclear mutants, demonstrate that the proteins activate translation by interacting with the 5' untranslated region of the COX 3 transcript (Muller et al., 1984; Costanzo and Fox, 1986; Costanzo et al., 1986; Costanzo and Fox, 1988). The suppressors are mtDNA rearrangements that generate novel chimeric COX 3 mRNAs that retain the entire COX 3 coding region, but have 5' untranslated leaders derived from other mitochondrial genes. These petite (rho$^-$) mtDNAs are retained in a heteroplasmic state with wild-type (rho$^+$) mtDNA, allowing the suppressor strains to be respiratory competent. The site of action of *PET494, 54* and *122* has been narrowed down to the 5' two-thirds (approximately 400 nucleotides) of the COX 3 leader by selecting as a suppressor to a *cbs1* mutation (see below), a mtDNA rearrangement that fuses this portion of the COX 3 leader to the COB structural gene. The translation of the COB gene is dependent on all three COX 3 translational activators (Costanzo and Fox, 1988). The nature of the suppressors suggest that the nuclear gene products do not have a post-translational function (such as stabilizing the COX 3 polypeptide), since the COX 3 coding region (or in the latter example, the COB coding region) remains unaltered in the suppressor mutations. Rather, the pet gene products appear to bind the 5' untranslated region of the COX 3 mRNA to activate translation. The suppressor mutations allow the COX 3 mRNA to be translated from a different leader sequence, and, therefore, independently of *PET494, 54* and *122* function. Additional evidence to support the hypothesis that *PET 494, 54* and *122* act directly on the COX 3 mRNA leader sequence (and not indirectly by, perhaps, activating another nuclear gene) was provided by demonstrating that all three proteins are located in mitochondria (Costanzo and Fox, 1986; Ohmen et al., 1988; Costanzo et al., 1989).

At least two of the three translational activators of the COB gene transcript, *CBS1* and *CBS2*, appear to act in a similar fashion to the COX 3 specific translation factors. That is, mitochondrial suppressor mutations of *cbs1* and *cbs2* mutants are fusions of the COB coding sequence with the 5' untranslated regions of other genes such as *oli1* (Rodel et al., 1985; Rodel, 1986; Rodel and Fox, 1987). *CBS1* and *CBS2* have also been immunologically localized to mitochondria (Michaelis and Rodel, 1990; Michaelis et al., 1991). Thus, the proteins activate translation of COB mRNA by interacting with its leader sequence. The *cbp6* gene product is also required for COB translation, although mutants of this gene have not been shown to be suppressible by COB leader substitution mutations (Dieckmann and Tzagoloff, 1985). A specific nuclear encoded translational activator of the COX 2 gene has also been identified. The *pet111* gene product, which has been localized to mitochondria (Strick and Fox, 1987), acts on the 54 nucleotide 5' untranslated leader of the COX 2 mRNA (Mulero et al., 1993).

The many mRNA specific translational activators that have been identified, suggest that this is an important level of nuclear control of mitochondrial gene expression. For instance, in yeast the regulation of mitochondrial gene expression in response to O_2 and glucose levels occurs primarily at the level of translation (Woodrow and Schatz, 1979; Falcone et al., 1983; Zennaro et al., 1985). Steady state levels of mitochondrial mRNAs vary little in response to glucose or oxygen levels. Mitochondrial protein levels under these conditions may be controlled indirectly by regulating, among other nuclear genes, those coding for mRNA specific translation factors. The *pet494* and *cbs1* gene products, for example, have been demonstrated to be regulated by glucose and oxygen levels (Marykwas and Fox, 1989; Forsbach et al., 1989).

The exact mechanism of action of the gene-specific translational activators is not known. They may perform functions analogous to general eukaryotic translation initiation factors (Grivell, 1989). Insight into how the proteins act may be derived from analysis of second site suppressors of mutations in these genes. The COX 3 translation factor *PET122* has been analyzed in this way. Two nuclear suppressors of *pet122* mutations have been characterized as small subunit ribosomal proteins, *PET123* and *MRP1* (Haffter et al., 1990; McMullin et al., 1990; Haffter et al., 1991). *Pet123* and *mrp1* mutations can only suppress a specific allele of *pet122* (a carboxy-terminal truncation); complete disruptions of *pet122* cannot be suppressed, indicating that the suppressors do not bypass *PET122* function. This suggests that the ribosomal proteins and *PET122* interact directly. The *PET122* translation factor, therefore, appears to mediate its affects by physically associating with the small subunit ribosomal proteins (Haffter et al., 1991). This may be the mechanism by which other mRNA specific translation factors act.

Several nuclear gene products exert their effects on mitochondrial gene expression at the post-translational level. Post-translational regulation can involve processing primary translation products, stabilizing proteins or assembling proteins into a quaternary complex. An example of a mutant defective in N-terminal processing of the COX 2 protein into its mature form has been identified by Pratje *et al.* (1983). A temperature sensitive mutant, *ts2858*, accumulates the precursor of COX 2, carrying the 15 amino acid presequence of the protein, at the restrictive temperature. Surprisingly, the mutant is also defective in processing the nuclearly encoded cytochrome b_2, but is not generally defective in removing presequences of other mitochondrial proteins coded for by the nucleus (Pratje and Guiard, 1986).

The *sco1* gene product is essential for assembly and stability of the COX 1 and COX 2 subunits of cytochrome oxidase. *Sco1* mutants generate normal levels of mature COX 1 and COX 2 transcripts, but are deficient in the respective proteins (Schulze and Rodel, 1988). Krummeck and Rodel (1990) performed pulse-chase experiments and demonstrated that COX 1 and COX 2 are translated, but rapidly degraded in *sco1* deletion mutants, indicative of a post-translational defect. *SCO1* is a mitochondrial membrane associated protein, proposed to function in assembly of COX 1 and COX 2 into a pre-complex, which then allows these subunits to be integrated into holocytochrome *c* oxidase (Schulze and Rodel, 1989). Mutants of the *cbp3* gene appear to be deficient in assembly of coenzyme QH_2-cytochrome *c* reductase (complex 3 of the electron transport chain). Mutants have reduced levels of cytochrome *b*, iron-sulfur protein and several other core subunits, but can synthesize all of these proteins (Wu and Tzagoloff, 1989). *CBP3* is a mitochondrial membrane protein, but has not been detected in purified complex 3 preparations, implying it is not a structural subunit of the complex. The protein may be involved in folding or assembly of the structural subunits of the complex, or may function in post-translational modification of one of the subunits, perhaps attaching the heme prosthetic group to cytochome *b*, or the iron-sulfur centre of the Rieske protein (Wu and Tzagoloff, 1989).

It appears that assembly of the F_0/F_1 ATPase complex requires several nuclear encoded proteins. However, as for the *cbp3* mutants, it is difficult to distinguish whether mutants of these proteins have a primary defect in assembly of the complex, or functions required for proper folding or modification of a specific subunit of the complex. A defect in the latter could indirectly disrupt assembly of a complex. Nevertheless, mutants of the *atp10* gene disrupt assembly of the F_0 sector of the F_0/F1 ATPase (Ackerman and Tzagoloff, 1990a), whereas mutants of *atp11* and *atp12* do not assemble the F_1 sector (Ackerman and Tzagoloff, 1990b; Bowman et al., 1991; Ackerman et al., 1992). Neither *ATP10*, *ATP11* or *ATP12* can be detected in purified preparations of the ATPase complex, and they do not have any sequence homologies with identified subunits of yeast, bovine or *E. coli* (Ackerman and Tzagoloff, 1990 a and b); thus, they probably are not subunits of the complex.

Neurospora crassa

In comparison to *S. cerevisiae*, nuclear control of mitochondrial gene expression in *Neurospora crassa* has not been well characterized. This is primarily due to the nature of the organism.

Neurospora is an obligate aerobe; mutations which impair essential functions in respiration are most often lethal, whereas similar mutants in yeast can be maintained on medium containing a fermentable carbon source. Hence, mutations of many mitochondrial functions in *Neurospora* have not been obtained. Nevertheless, many of the nuclear genes perfoming mitochondrial functions have been identified by initially selecting for mutants that exhibit slow growth (Edwards et al., 1973; Bertrand et al., 1977; Pittenger and West, 1979). Such mutants are then further examined for deficiences in mitochondrial respiratory functions. Preliminary analysis involves examining the cytochrome absorption spectra of the mutants. This tests for the presence of functional cytochromes *aa3*, *b* and *c*. Absence or reduced levels of one or more of these cytochromes indicates defects in the electron transport chain (Fig. 1), which in turn, suggests possible effects on mitochondrial gene expression.

Only a few nuclear genes that code for components of the mitochondrial general translation system have been isolated. These include the *cyt-21* (Kuiper et al., 1988) and *mrp-3* (Kreader et al., 1989), which encode ribosomal proteins. Genes coding for mitochondrial leucyl-tRNA synthetase (Chow et al., 1989) and tyrosyl-tRNA synthetase (*cyt-18* gene; see below) have been characterized. Furthermore, the *cyt-20* gene codes for both the mitochondrial and cytosolic valyl-tRNA synthetases (Kubelik et al., 1991).

The *cya-5* gene reported in this study is the only nuclear gene so far characterized in *Neurospora* that appears to affect the expression of a specific mitochondrial gene (COX 1). Most nuclear mutants affecting mitochondrial function appear to have deficiencies in more than one of the mitochondrial cytochromes, implying that they define genes encoding products that affect the expression of several mitochondrial genes (Bertrand et al., 1977; Pittenger and West, 1979). The most well studied of these mutants define three genes (*cyt-4, cyt-18* and *cyt-19*) that function in splicing of several group 1 introns in *Neurospora* mitochondria, suggesting that they encode general functions of intron splicing. Interestingly, *CYT4* and *CYT18* (and perhaps *CYT19*) also have other important functions in the cell, as do many mitochondrial splicing factors characterized in *S. cerevisiae* (see above). The CYT-4 protein appears to act as a protein phosphatase (Turcq et al., 1992) that functions not only in intron splicing, but 5' and 3' end processing of many mitochondrial mRNAs and tRNAs (Garriga et al., 1984; Dobinson et al., 1989).

The *cyt-18* gene product has been characterized as a mitochondrial tyrosyl-tRNA synthetase (Akins and Lambowitz, 1987). That *CYT-18* has a secondary function in group 1 intron splicing has been confirmed by demonstrating that it is essential for splicing *in vitro* (Garriga and Lambowitz, 1986; Guo et al., 1991). As demonstrated for the *CBP2* protein in yeast (see above), *CYT-18* aids in splicing by interacting with the catalytically active core region of group 1 introns and stabilizing secondary structures required for splicing (Cherniack et al., 1990; Guo et al., 1991; Mohr et al., 1992).

Object of this study

This study focuses on characterization of the *cya-5*$^+$ nuclear gene of *Neurospora crassa*, which affects the expression of the mitochondrially encoded subunit one of cytochrome *c* oxidase. The *cya-5* mutant was originally isolated using a procedure previously described to select for mutants of mitochondrial respiratory functions (Edwards et al., 1973; Bertrand et al., 1977). This procedure selects for strains displaying slow growth and the inability to reduce tetrazolium. Analysis of the cytochrome absorbance spectra of the mutant revealed that it lacked cytochrome *aa3* and had a two-fold increase in cytochrome *c* compared to wild-type strains (Nargang et al., 1978). In agreement with the fact that cytochrome *aa3* could not be spectrally detected, the mutant showed very low levels of cytochrome oxidase activity. As a consequence of the cytochrome oxidase deficiency, *cya-5* is highly dependent on the cyanide insensitive alternate oxidase pathway of respiration (Fig. 1; Lambowitz and Slayman, 1971). Further analysis revealed that the cytochrome oxidase deficiency in *cya-5* is specifically a result of absence of subunit 1 of the enzyme (Nargang et al., 1978).

The *cya-5*$^+$ gene has been cloned and in this study the sequence of the gene is reported. The coding region of the gene was confirmed by obtaining cDNA clones of the gene. Although the complete cDNA has not been obtained, sequence obtained from genomic and partial cDNA clones suggest that the gene encodes a

1136 amino acid protein. The protein appears to be expressed at very low levels as suggested by codon usage analysis of the DNA sequence, and by Northern analysis, in which the *cya-5*$^+$ transcript was not detected. The defect in COX1 gene expression is at the post-transcriptional level in *cya-5*, since COX1 mRNA was detected at normal levels in Northerns of mitochondrial RNA. Therefore, the *cya-5*$^+$ gene codes for a translational or post-translational factor affecting COX1 expression.

Materials and Methods

N. crassa strains and culture conditions

The strains of *N. crassa* used in this study are described in Table 1. The *cya-5*$^+$ strain used is referred to as NCN10. The mutant strain *cya-5-34, a* (NCN5) was derived from a single ascospore isolate of a cross between the original *cya-5* mutant isolate and a wild-type strain, 50*a* (Nargang et al., 1978). The *cya-5, nic-1, al-2*, strain (NCN80) is derived from a single ascospore isolate of a cross between NCN5 and NCN10. The strain NCN45 carries the extranuclear mutation *[mi-3]* (Lemire and Nargang, 1986). The NCN69 strain is a *cyt-2-1* mutant (Drygas et al., 1989).

Cultures of *N. crassa* were grown in either liquid or solid Vogel's medium (see Appendix) containing appropriate supplements (Davis and de Serres, 1970). For conidia generation, cultures were grown on Vogel's medium (see Appendix) solidified with 1.5% (w/v) agar in 250 ml erlenmeyer flasks. The flasks were incubated at 30°C until sufficient mycelial growth had occured on the surface of the medium. The flasks were then placed at room temperature in the light to allow the cultures to conidiate. For starting liquid cultures, the conidia were harvested by suspending them in sterile distilled water. Mycelial fragments were removed by filtering the conidial suspension through sterile cheesecloth, and the conidia were innoculated to a final concentration of 0.5-1 x 10^6/ml.

E. coli strains and culture conditions

The *E. coli* strains used in this study are described in Table 2. The strain JM83 was used to maintain recombinant clones of the pUC19 plasmid. Strain JM103 was used to propagate M13 clones. Strain XL1-Blue was used for amplification of a *N. crassa* wildtype cDNA library, and obtaining plasmid subclones of the lambda ZAP vector by the automatic excision protocol.

Most *E. coli* strains were grown in liquid or solid (1.5% agar) L-broth medium containing the appropriate antibiotics (see Appendix). Overnight cultures of JM103 were grown in DM salts (Davis and Mingioli, 1950) containing 10 µM thiamine and 0.5% glucose (see Appendix). Cultures of XL1-Blue were grown overnight in TB or LB broth (see Appendix) supplemented with 0.2% maltose and 10 mM $MgSO_4$. Before addition of lambda phage, these cells were centrifuged in a SS-34 rotor (Sorvall) at 3000 rpm for 10 minutes and resuspended in 10 mM $MgSO_4$ to an $O.D._{600}=0.5$.

For long term storage of *E. coli* strains, glycerol stocks were prepared as described by Maniatis *et al.* (1982). Sterile 50% glycerol was added to a final concentration of 15% to cells freshly grown to saturation.

Plasmid DNA vectors

Plasmids pUC19, pBS, and pBluescript were used as cloning vectors (Table 3). The recombinant plasmids used in this study are listed in Table 4.

E. coli transformation

E. coli transformation was performed using a procedure described by Mandel and Higa (1970). A fresh overnight culture was prepared in 10 ml of L-broth. A flask of 25 ml of L-broth was inoculated with 250 µl of the overnight culture. The flask was incubated at 37°C with vigorous shaking until the culture reached mid-log phase (OD at 600 nm of 0.5). The cells were pelleted in a SS-34 rotor (4000 rpm for 5 minutes at 4°C), and the supernatant discarded. The pellet was resuspended in 10 ml of cold, sterile 10 mM $CaCl_2$, and centrifuged again in a SS-34 rotor (4000 rpm for 5 minutes at 4°C). The cells were then resuspended in 0.5-1 ml of cold, sterile 30mM $CaCl_2$ and left on ice for 30 minutes. The ligation mixture was added to the cells, which were then incubated for another 30 minutes. The transformation mixture was heat shocked at 42-45°C for 2 minutes before adding 2 volumes of L-broth. The cells were incubated at 37°C for 30 minutes, and 25 to 150 µl aliquots were plated on selective media. The plates were incubated at 37°C for 16-24 hours.

M13 transfection

Cells in preparation for M13 transfection (JM103 strain) were treated as described in the procedure for bacterial transformation. For each transfection, 0.1-1 µg of DNA was added to 100-250 µl of competent cells, and incubated on ice for 30 minutes. Aliquots of 25-150 µl of the transformation mixture were plated on L-agar plates in soft agar (0.7%) containing 250 µl of lawn cells, 10 µl of IPTG (25 mg/ml), and 50 µl of X-gal (25 mg/ml in N,N-dimethylformamide) for each plate. The plates were incubated at 37°C for at least 9 hours.

Isolation of M13 single-stranded DNA

A plaque generated from the transfection procedure (see above) was used to inoculate a 25 ml L-broth flask which had been inoculated with 100 µl a fresh JM103 overnight culture. The culture was grown for 8 to 12 hours at 37°C with rapid shaking. The cells were pelleted by centrifuging in a SS-34 rotor (15000 rpm for 30 minutes at 4°C). The supernatant was decanted into a another tube, and 6 ml of polyethylene glycol solution (10% PEG 8000, 2.5 M NaCl) was added. The mixture was left on ice for 30 minutes, or at 4°C overnight. The sample was then centrifuged in a SS-34 rotor (12000 rpm for 20 minutes at 4°C). The supernatant was discarded, and the tube was left upside down to remove as much PEG as possible. The pellet was resuspended in 650 µl of phenol extraction buffer (300 mM NaCl, 100mM Tris-Cl pH7.9, 1mM EDTA), and 10 µl of each of

10% SDS and protease K (2 mg/ml) were added. The reaction mixture was incubated at 37 °C for 30 minutes. This was followed by two phenol extractions and one chloroform/isoamyl alcohol (1:24) extraction. The DNA was precipitated with 95% ethanol and dried in a dessicator. The pellet was resuspended in 50 µl of distilled water and 1-3 microlitres was run together with a single stranded DNA standard (DNA isolated from a blue plaque) on a 0.8% agarose gel, to determine which isolates contained single stranded DNA inserts of the appropriate size.

Rapid plasmid DNA Isolation

Rapid DNA isolation was performed using an alkaline lysis procedure modified from Maniatis *et al.* (1982). Cells from 5 ml of *E. coli* overnight culture (containing the appropriate antibiotic; see Appendix) were collected in 1.5 ml aliquots into eppendorf tubes. The cells were centrifuged for 1 minute, and the supernatant was discarded. The pellet was resuspended by vortexing in 100 µl of an ice-cold solution consisting of 50 mM glucose, 10 mM EDTA, 25mM Tris-Cl (pH 8.0) and 4 mg/ml lysozyme. The mixture was incubated for 5 minutes at room temperature. A freshly prepared, ice-cold solution of 0.2 N NaOH, 1% SDS (200 µl) was then added, and the suspension was mixed by inverting the tube rapidly two or three times (avoiding vortexing). The tube was incubated on ice for 5 minutes. This was followed by addition of 150 µl of an ice-cold solution of sodium acetate (prepared by mixing 60 ml of 5M sodium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of H_2O). The mixture was vortexed gently and stored on ice for 5 minutes. The mixture was then centrifuged for 5 minutes in an eppendorf centrifuge at 4°C. The supernatant was transferred to a new tube, and an equal volume of phenol/chloroform (1:1) was added. The mixture was vortexed and centrifuged for 2 minutes in an eppendorf centrifuge. The supernatant was transferred to a fresh tube, and chloroform extracted. Two volumes of 95% ethanol at room temperature was added to the supernatant, and the mixture was vortexed. This was left to stand at room temperature for 2 minutes, and was then centrifuged for 5 minutes in an eppendorf centrifuge at room temperature. The supernatant was decanted and the tube was allowed to dry. The pellet was washed with 1 ml of 70% ethanol and dryed in a dessicator. The pellet was resuspended in 50 µl of distilled water, and 2-5 µl was used in each restriction digest. To each digest, 1µl of RNase A (2mg/ml) was added to remove contaminating RNA. The DNA was then analyzed by electorphoresis on agarose gels.

Large-scale plasmid DNA isolation

Plasmid DNA was isolated by a modification of a procedure described in Maniatis *et al.* (1982). Two flasks, each containing 1 litre of L-broth supplemented with ampicillin (see Appendix), were inoculated with a fresh overnight culture of cells harboring the plasmid of interest. These cultures were incubated overnight at 37°C with rapid shaking. The cultures were harvested and then centrifuged in a Sorvall GS-3 rotor (5000 rpm for 5 minutes at 4°C), and the supernatant discarded. The cells were resuspended in 20 ml of STE buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.8 at 4°C, 1mM EDTA), and centrifuged in a GS-3 rotor (5000 rpm for 5 minutes at 4 °C). The supernatant was discarded, and the pellet was resuspended in 10 ml of 10% sucrose buffer (10% sucrose, 50 mM Tris-HCl, pH8.0 at 4°C). The cells were then treated with 2 ml of a freshly prepared solution of lysozyme (10 mg/ml in 0.25 M Tris-HCl, pH 8.0 at 4°C), and 8 ml of 0.25 M EDTA, pH 8.0. The solution was mixed well by inversion and incubated on ice for 10-30 minutes. This was followed by the addition of 10% SDS (4 ml) and 6 ml of 5 M NaCl. The solution was mixed gently and placed on ice for 1 hour, mixing occasionally. The mixture was centrifuged in a SS-34 rotor (18000 rpm for 30 minutes at 4°C), and the supernatant was decanted to a new tube. Isopropanol (0.6 volumes) was added and the mixture was left on ice for 10 minutes. The nucleic acids were pelleted by centrifuging in a SS-34 rotor (12000 rpm for 10 minutes at 4°C), and the supernatant was discarded. Any droplets of isopropanol were carefully wiped off and the pellets were resuspended in a total volume of 3 ml of distilled water. The insoluble material was pelleted by centrifugation in a SS-34 rotor (10000 rpm for 5 minutes at 4°C), and the supernatant was decanted to a new tube. For each ml of solution, 1g of CsCl was added and mixed until it completely dissolved. Ethidium bromide (600 µl of 10 mg/ml solution) was added, and the solution was left in the dark at room temperature for 15 minutes. This was followed by a 15 minute centrifugation in a SS-34 rotor (15000 rpm at 4°C). The supernatant was transferred to VTi65 quick-seal tubes (Beckman) and the plasmid DNA was banded by equilibrium-density centrifugation in a VTi65 rotor (Beckman) at 54000 rpm for at least 6 hours at 20°C. The plasmid DNA was collected (corresponding to the lower ethidium bromide stained band), and transferred to new quick-seal tubes. The tubes were filled with CsCl (from a 1 g/ml solution) and a few drops of ethidium bromide (10 mg/ml) from a Pasteur pipette. The plasmid DNA was banded a second time in a VTi65 rotor (54000 rpm for 6 hours at 20°C). The DNA was collected and extracted 4 to 5 times with salt-saturated isopropanol to remove ethidium bromide, and dialyzed against TE buffer (10 mM Tris-Cl pH 7.8, 1mM EDTA) to remove CsCl. The sample was then treated with 20 µl of RNase A (10 mg/ml) and 20 µl of Protease K (3 mg/ml), and incubated at 37°C for 30 minutes. The mixture was phenol extracted twice and chloroform extracted once. This was followed by the addition of 1/2 volume of 7.5 M ammonium acetate (pH7.5) and 3 volumes of 95% ethanol. The DNA was allowed to precipitate at -20°C for at least 30 minutes, and was pelleted by centrifugation in a microcentrifuge for 15 minutes. The pellet was dried in a dessicator, and was resuspended in 500 µl to 1 ml of distilled water. The concentration of DNA was determined in a spectrophotometer (an O.D. of 1 at 260 nm = 50 µg/ml of DNA), and approximately 0.5 µg was digested with an appropriate restriction enzyme and electrophoresed on an agarose gel.

Neurospora transformation

Transformation of *N. crassa* sphaeroplasts was performed using the procedure of Schweizer *et al.* (1981) with modifications described by Akins and Lambowitz (1985). For sphaeroplast formation, fresh *cya-5* conidia (less than 2 weeks old) were harvested, and 2.5-7.5 x 10^9 conidia were germinated at 30°C with gentle shaking (200 rpm) in each of three flasks containing 1 litre of 0.5x Vogel's (see Appendix). Germination of conidia was monitored by periodically examining 30 µl aliquots under a microscope, and determining the number of germinating conidia as a percentage of the total number of conidia in the field of vision. Incubation was continued until at least 80% germination was reached (about 6 hours), after which the conidia were harvested by centrifugation in a Sorvall GS-3 rotor at 5000 rpm for 10 minutes at 4°C. The conidia were then washed once with sterile water and twice with sterile 1M sorbitol. The final pellet was resuspended in 9 ml of 1M sorbitol, and the concentration of conidia was determined and adjusted to approximately 4 x 10^8/ml. The suspension was transferred to a 250 ml Erlenmeyer flask and lysing enzyme (Sigma) was added to a final concentration of 3.5 mg/ml. The solution was incubated at 30°C with gentle agitation (100 rpm) for approximately 1 hour (time required for a >90% level of sphaeroplasting to be reached). The sphaeroplasts were spun down in a clinical centrifuge at the lowest speed for 10 minutes, and washed twice with sterile 1M sorbitol and once with sterile MCS buffer (10mM MOPS pH 6.3, 50mM calcium chloride, 1M sorbitol). The supernatant after each centrifugation was removed by gentle suction. The final pellet was resuspended in 8 ml of MCS, and the concentration of the suspension was determined and adjusted to 3 x 10^8/ml. For each ml of sphaeroplast solution, 13 µl of dimethylsulfoxide, 65 µl of 5 mg/ml Heparin and 275 µl of PMC (40% (w/v) PEG-4000, 10 mM MOPS pH 6.3, 50 mM calcium chloride) were added and gently mixed. Aliquots of 0.05 to 1 ml sphaeroplasts were stored at -70°C. Viability of the sphaeroplasts was tested by thawing an aliquot of the sphaeroplasts and plating a series of dilutions (100 µl of 10^{-3} - 10^{-6} dilutions) in top agar (see Appendix) onto Vogel's viability plates (see Appendix). Once thawed, the sphaeroplasts were never refrozen for subsequent use.

For each transformation, 0.5 µg of DNA was added to 100 µl of sphaeroplasts (a ratio of 5 x 10^6 viable sphaeroplasts/µg of DNA). Prior to addition, the volume of the DNA samples was adjusted with water to equal 60% of the volume of the sphaeroplasts. The mixture was incubated on ice for 30 minutes, after which time PMC was added to 10 times the volume of the sphaeroplasts and DNA. This mixture was left at room temperature for 20 minutes. The transformation mixture was then added to benomyl containing top agar (10 ml/plate; see Appendix), and plated onto benomyl containing transformation plates (see Appendix). The plates were incubated at 30°C, and fast growing colonies (rescued transformants) were visible after 48 hours. A background of non-rescued, benomyl resistant transformants was visible after approximately 72 hours. All operations were performed under sterile conditions.

Isolation of *N. crassa* total RNA

Total RNA was extracted by a modification of the procedure of Chirgwin *et al.* (1979). All glassware used for RNA isolation was baked at 180°C for at least 4 hours to inactivate RNases. For the same purpose, solutions were treated with 0.1% diethylpyrocarbonate (DEPC) over night and autoclaved. The presence of Tris inactivates DEPC. Therefore, solutions containing Tris were prepared in water that had been pretreated with DEPC. The final solutions were then autoclaved. Conidia from mutant and wild-type strains for RNA isolation were inoculated to a concentration of approximately 1x 10^6/ml in Vogel's medium (see Appendix) containing 2% sucrose and 0.2% Tween 80. The cultures were grown at 30°C, and mycelia from wild-type and mutant cultures were harvested by filtration after 16 hours and 30 hours, respectively. The mycelia were immediately frozen in liquid nitrogen, and ground to a powder in a mortar with acid washed, heat sterilized sand (1.5 g per 1 g of mycelium) and 7-10 volumes of guanidine solution (see Appendix). The suspension was centrifuged in a SS-34 rotor at 9000 rpm for 20 minutes at 4°C. The supernatant was saved and added to Ti 50 tubes, each containing 5 ml of CsCl solution (5.7 M CsCl, 0.1 M EDTA). The supernatant was gently layered onto the CsCl solution using a Pasteur pipette. The samples were centrifuged overnight in a Ti 50 3236 rotor at 44000 rpm at

20°C. The supernatant was decanted, and the RNA pellets were washed 3 times with water. The pellets were resuspended in a total volume of 3 to 4 ml of buffer containing 0.1% SDS and 25 mM EDTA for at least 5 hours. The solution was then phenol extracted once and chloroform extracted once. A solution of 10 M ammonium acetate was added to a final concentration of 2.5 M, along with 2 volumes of 95% ethanol. The RNA was left to precipitate at -20°C overnight. The sample was then centrifuged in a clinical centrifuge (maximum speed, 1hour) to collect the pellet. The pellet was washed once with 70% ethanol and dryed under vacuum. The RNA was resuspended in distilled water and stored at -70°C.

Poly A RNA isolation

Poly A RNA was purified from total RNA by a modified batch isolation procedure using oligo-dT cellulose. Glassware and solutions used for poly A RNA isolation were treated to remove RNases as described for the total RNA isolation (see above). The oligo-dT cellulose (0.25 g) was initially treated with approximately 10 ml of 0.1 M NaOH in a disposable, sterile falcon tube. The suspension was inverted gently over a 5 minute period, and then allowed to settle with no agitation for another 5 minutes. The oligo-dT cellulose was gently spun down in a clinical centrifuge for 2 minutes, and the supernatant decanted. The cellulose was similarily treated with water once and poly A loading buffer (see Appendix) twice. Total RNA (2.5 mg) was heated at 65°C for 10 minutes and set on ice. An equal volume of 2X poly A loading buffer was added to the total RNA sample to make the final concentration equal that of the 1X loading buffer. The RNA was added to oligo-dT cellulose, which was resuspended in 10 ml of fresh poly A loading buffer, and the mixture was gently inverted over a period of 30 minutes. It was allowed to settle for 5 minutes, and was then gently spun down in a clinical centrifuge for 2 minutes. The poly A loading buffer was decanted. The oligo-dT cellulose was similarily treated with middle wash buffer twice (see Appendix), except mixing was continued for only 10 minutes. Bound poly A RNA was eluted by adding 1 ml of distilled water and 1 ml of elution buffer (see Appendix), mixing gently (no inversion), and setting still for 10 minutes. The mixture was gently spun down for 2 minutes in a clinical centrifuge, and the supernatant was transferred to a glass Corex tube. A solution of 10 M ammonium acetate was added to a concentration of 3 M, along with 3 volumes of 95% ethanol, and the poly A RNA was allowed to precipitate at -20°C overnight. The next day, the precipitate was collected by centrifugation in a microfuge at 10 000 rpm for 1 hour at 4°C. The pellet was washed with 70% ethanol, and dryed under vacuum. The RNA was resuspended in distilled water, and stored at -70°C.

RNA electrophoresis and Northern transfer

The preparation of formaldehyde gels and RNA samples for electrophoresis was carried out as described by Maniatis *et al.* (1982) with minor modifications. The gels were prepared by melting 2 g of agarose (final concentration of 1%) in 144.3 ml of sterile, DEPC treated water by boiling. This was cooled to 60°C, and 35.7 ml of 37% (12.3 M) formaldehyde (final concentration of 2.2 M) and 20 ml of 10X MOPS buffer (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA pH 7.0) were added and mixed well. The gel was poured into a gel tray and allowed to cool and solidify for at least 1 hour before loading. RNA samples were prepared by mixing 4.5 µl of RNA (up to 20 µg), 1 µl of 10X MOPS buffer, 3.5 µl of 37% formaldehyde and 10 µl of deionized formamide, and incubating at 65°C for 15 minutes. The samples were chilled on ice and briefly centrifuged. This was followed by the addition of 1 µl of ethidium bromide (10 mg/ml) and 2 µl of formaldehyde gel loading buffer (50% glycerol, 1mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol, FF). The samples were then immediately loaded onto the gel. Samples were electrophoresed at 25 V in 1X MOPS buffer for approximately 18 hours (bromophenol blue migrates ~10 cm into the gel).

Northern blotting was carried out as described by Fourney *et al*. (1988). The gel was photographed and then soaked for two 20-minute periods in 10X SSC at room temperature with gentle shaking. A Biotrans nylon membrane (ICN Biochemicals, Inc.) was immersed in distilled water for 5 minutes followed by a 5-minute soak in 10X SSC, before setting up the transfer. Prior to transfer, the nylon membrane, 6 sheets of Whatman 3 MM paper and a stack of paper towels were carefully cut to the same size as the gel. The transfer was set up as follows: a sponge (of approximately the same size as the gel) was placed in a glass dish, and was overlayed with 3 sheets of Whatman 3 MM paper. The gel was layed on top of the Whatman paper, and the nylon membrane was then carefully placed on the gel, making certain that there were no air bubbles between the gel and the membrane. This was followed by another 3 pieces of Whatman 3 MM paper, and then an approximately 10 cm stack of paper towels. A 1 kg weight was then placed on top of the whole apparatus. Transfer was allowed to take place for at least 17 hours. After approximately 3 hours of transfer, soaked paper towels were replaced with dry ones. This was repeated approximately 12 hours later. Upon completion of transfer, the apparatus was disassembled, and the membrane was allowed to air dry. The membrane was then baked at 80°C for 1 1/2 hours, after which it was ready for hybridization.

Southern transfer and plaque lifts

Southern transfers were performed by a modification of the procedure described by Southern (1979). After restriction fragments or PCR products were electrophoresed on a 0.8% agarose gel, the gel was submerged in denaturing solution (1.5 M NaCl, 0.5 M NaOH) with gentle shaking for 30 minutes. The gel was then placed in neutralizing solution (3 M sodium acetate, pH 5.5) and gently agitated for another 30 minutes. It was now ready for transfer. The gel was placed on saran wrap and a piece of Biotrans nylon membrane (ICN Biochemicals, Inc.) was placed on top of the gel. This was followed by 3 pieces of Whatman 3 MM paper and then a stack of paper towels. Finally, a 1 kg weight was placed on top of the paper towels. The membrane, Whatman paper and paper towels were carefully cut to the same size as the gel prior to setting up transfer. This was allowed to stand at least 4 hours to allow transfer of DNA from the gel onto the membrane. The apparatus was then disassembled and the membrane was air-dried and baked at 80°C for 2 hours.

Plaque lifts were carried out as described in protocols for Biotrans nylon membranes (ICN Biomedicals, Inc.). A nylon membrane was carefully placed on the agar surface and marked with a sterile needle for later orientation of plaques with signals obtained from autoradiograms. After 1 minute, the membrane was removed and placed for 3 minutes, plaque side up, on Whatman 3 MM paper soaked with 10% SDS. The membrane was then placed on Whatman paper soaked with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 minutes, followed by neutralizing solution (3M sodium acetate pH 5.5) for another 5 minutes. The membrane was then air-dried for 30 minutes and baked at 80°C for 1 hour. Membranes were washed with a solution containing 50 mM Tris-Cl, pH 8.0, 1M NaCl, 1mM EDTA, 0.1% SDS at 42°C for 1 hour to remove any media or bacterial debris before hybridization.

Northern and Southern hybridizations

Hybridizations of filters from Northerns, Southerns and plaque lifts were performed as described in the protocols for Biotrans nylon membranes (ICN Biomedicals, Inc.). The same solution was used for pre-hybridization and hybridization reactions. It consisted of 5X Denhardt's (see Appendix), 5X SSC (see Appendix), 50mM sodium phosphate pH 6.5, 0.1% SDS, 250 μg/ml of nonhomologous DNA and 50% deionized formamide. Pre-hybridization reactions were done in sealed bags in a water bath set at 42°C for at least 1 hour. For hybridization reactions, denatured probe (see Radiolabelling DNA) was added to the hybridization solution prior to incubation with filters. The reaction was allowed to proceed for 16 to 24 hours at 42°C. After the hybridization reaction was completed, membranes were washed 4 times in 2X SSC, 0.1% SDS at room temperature with shaking, and twice in 0.1X SSC, 0.1% SDS at 50-65°C (depending on the stringency required) for 15 to 30 minutes. Following the wash, the membranes were subjected to autoradiography.

To remove a probe for subsequent re-hybridization experiments, Northern filters were boiled in 0.1X SSC, 0.1% SDS for 30 minutes. Southern and plaque lift filters were treated with 0.2 N NaOH at 65°C for 15 minutes, followed by a rinse with 0.2 M Tris-Cl (pH 7.5), 0.1% SDS, 0.1X SSC at 65°C for 15 minutes.

Radiolabelling of DNA

Restriction fragment probes for hybridizations were made using the method described by Feinberg and Vogelstein (1983; 1984). Labelling was performed using random oligonucleotide primers. The reagents for the reaction were either provided by a kit obtained from Pharmacia (Oligolabelling Kit) or obtained individually (see Appendix for description of oligolabelling buffer). DNA (50 to 100 ng in 34 microlitres) for labelling was denatured by boiling for 2-5 minutes and quickly cooled on ice. Then 10 µl of oligolabelling buffer (supplied by a kit or made as described in Appendix), and 5 µl (50 µCi) of [alpha-^{32}P]dCTP (3000 Ci/mmol) was added to the denatured DNA, followed by 6 units (1 µl) of Klenow fragment of *E. coli* DNA polymerase I. The reaction was allowed to proceed at 37°C for 45 minutes to overnight. Labeled DNA was purified from unincorporated nucleotides by spin-column chromatography as described by Maniatis *et al.* (1982), using Sephadex G-50. To determine the degree of incorporation of the radioactivity into labelled DNA, 1 µl samples of the probe (before passing through a Sephadex G-50 column) were spotted onto two DE81 ion exchange Whatman filter papers. One of the papers was washed with 10 ml of 0.3 M ammonium formate solution, which removes unincorporated nucleotides. The filters were placed in vials containing scintillation fluid (Amersham), and were counted in a Beckman LS7500 liquid scintillation counter. The number of counts in the washed and unwashed filters was compared, and the difference reflected the efficiency of incorporation of nucleotides during the labelling reaction. The amount of probe to be used in the hybridization reaction and its specific activity could then be calculated. Before adding the probe to hybridization solution, it was denatured by boiling for 5 minutes, and then quickly cooled on ice.

DNA sequencing

The dideoxy chain termination method (Sanger et al., 1977) was used in determining the DNA sequence. Sequence was obtained either directly from double stranded DNA template, or the DNA to be sequenced was cloned into the M13 phage vectors mp18 and mp19 (Yanisch-Perron et al., 1985) for the generation of single stranded template DNA. Reagents and protocols were obtained from a Sequenase® Version 2.0 kit (United States Biochemical). In sequencing single stranded DNA, 1 μl (10-30 ng of a 17-mer) primer was mixed with 2 μl of 5X reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl2, 250 mM NaCl) and 7 μl of template DNA (1-2 μg) in an eppendorf tube. The tube was heated at 65°C for 2 minutes, and allowed to cool slowly to room temperature over a period of about 30-45 minutes. To the annealed template and primer, the following was added: 1μl of 0.1 M dithiothreitol, 2μl of labelling mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP) that had been diluted 4-fold in water, 0.5 μl (5 μCi) of [alpha-^{35}S]dATP and 2 μl of the enzyme Sequenase® Version 2.0 which had been diluted 1:8 in enzyme dilution buffer (10 mM Tris-HCl pH 7.5, 5 mM dithiothreitol, 0.5 mg/ml BSA). The labelling reaction was incubated for 2-5 minutes at room temperature. During this time, 2.5 μl of each of the ddATP, ddCTP, ddGTP and ddTTP termination mixes (see Appendix) were added to 4 eppendorf tubes labelled A, C, G and T, respectively, and were pre-warmed at 37°C for 2 minutes. When the labelling reaction was complete, 3.5 μl of this reaction was transferred to each of the 4 tubes labelled A, C, G and T. The contents of the tubes were mixed thoroughly and incubated in a temp-bloc at 37°C for 5 minutes. Finally, the reactions were terminated by adding 4 µl of stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF) to each tube with mixing. When regions of compression in the DNA sequence were encountered, labelling and termination mixes containing dITP in place of dGTP were used to sequence the region. The samples were either loaded on a gel immediately (see below) or stored at -20°C for future loading.

When sequencing double stranded template, isolated DNA (see Rapid Plasmid DNA Isolation) was first PEG precipitated to remove any remaining RNA from the preparation after RNase treatment. The DNA preparation was treated with an equal volume of 13% PEG-8000 and 1/4 volume of 4 M NaCl and incubated on ice for 30 minutes. It was then centrifuged in a microcentrifuge for 10 minutes at 4°C, and the supernatant carefully decanted. The pellet was washed with 70% ethanol and dried in a dessicator. The DNA was now ready to sequence. The DNA (3-5 µg) was denatured in 0.2 M NaOH, 0.2 mM EDTA for 30 minutes at 37°C. The mixture was neutralized by adding 0.1 volumes of 2 M ammonium acetate (pH 4.8), and the DNA was precipitated with 3 volumes of 95% ethanol at -70°C for 15 minutes. The DNA was pelleted in a microcentrifuge (10 minutes, 4°C), and washed with 70% ethanol. The pellet was dried and resuspended in 7 µl of distilled water. The annealing, labelling and termination reactions were carried out as described for sequencing single stranded DNA. An alternate and more efficient procedure for sequencing double stranded DNA with the Sequenase kit (described by Hsiao, 1991) was also used. In this procedure, 1 µl (10-20 ng of a 17mer) of sequencing primer is added directly to 5 µl (1-2 µg) of non-denatured template. This is followed by the addition of 1 µl of 1N NaOH with mixing. The solution is incubated at 37°C for 10 minutes, and 1 µl of 1N HCl is added to neutralize the mixture. Then 5X reaction buffer (2 µl) is added, and the annealing reaction is incubated at 37°C for 5 minutes. The remaining steps in the procedure are identical to those described above.

The sequencing reactions were loaded onto 6% (w/v) polyacrylamide denaturing gels (see Appendix). Prior to loading, the reactions were boiled in water for 3 to 5 minutes to denature the single stranded DNA products. Electrophoresis was performed at 25-30 watts (constant power) for short gels (40 cm x 20 cm x 0.3 mm) and 50-55 watts for long gels (80 cm x 20 cm x 0.3 mm), in 0.1 M Tris-borate pH 8.3, 2 mM EDTA. After electrophoresis, sequencing gels were dried at 80°C in a Slab Dryer (BioRad) Model 483, and autoradiographed at room temperature for at least 16 hours. The film used for autoradiography was either Kodak XAR-5 or Fuji RX film.

Isolation of *cya-5*$^+$ cDNA from a lambda ZAP library

Two wild-type *N. crassa* cDNA libraries constructed in the lambda ZAP/EcoRI vector (Stratagene) were generously provided by

Dr. Matthew Sachs (Stanford, California). One library was constructed from poly A RNA isolated from mycelial culture, while the second library was constructed from poly A RNA isolated from a culture of germinating conidia. The procedure used to isolate *cya-5*$^+$ cDNA clones from the libraries was described in a manual provided by Stratagene. Approximately 300 000 phage from each library were screened. This was performed by diluting the phage stock from each library (2 x 10^{10} pfu/ml) 10^{-5} in SM buffer (see Appendix) and plating approximately 50 000 phage onto each of 12 large (150 mm x 15 mm) LB plates (see Appendix) that were one day old. The plates were incubated at 37°C for 8 to 10 hours until plaques just appeared (to avoid the plaques becoming confluent). They were then left at 4°C for at least 2 hours, before transferring the plaques onto Biotrans (ICN Biomedicals, Inc.) nylon membranes. Plaque lifts were performed as described on page 47. Filters were hybridized to a ~6.4 kb SphI-XmaI probe containing the *cya-5*$^+$ gene. The density of plaques in the first round of plating did not allow purification of a single plaque corresponding to a positive signal from the hybridization reaction. Therefore, the area of the initial plates corresponding to a signal were further purified and rescreened until single well isolated plaques correlated with a hybridization signal. This required 3 rounds of plating. Plaques were picked with a sterile scalpel and suspended in 0.5 ml of SM buffer containing chloroform. The suspension was vortexed and left overnight at 4°C. Each phage stock was titered and the cloned inserts carried on the phagemid pBluescript SK (M13-) were removed from lambda ZAP phage by the *in vivo* excision process (Fig. 2). This process allows one to obtain cDNA inserts that are already subcloned into a phagemid vector, and is carried out as follows. The recombinant lambda ZAP genome consists of the pBluescript vector carried within lambda arms. The cDNAs are, in turn, carried within the cloning site of the pBluescript sequences. The pBluescript and cDNA insert sequences are flanked by sequences that can serve as the sites of initiation and termination for bacteriophage f1 replication (Fig. 2). An *E. coli* strain (XL1 Blue) is simultaneously infected with the lambda ZAP recombinant phage and a f1 (or M13) helper bacteriophage. The helper phage produces proteins that recognize the f1 phage initiation and termination signals within the lambda vector and replicate one strand of the DNA between these sequences. The resulting single stranded DNA molecule, which includes sequences of the pBluescript phagemid and the cDNA insert, is circularized by another protein (gene II product) produced by the helper phage. The circularized DNA is packaged by f1 (or M13) coat proteins and released from *E. coli*. *E. coli* cells infected by packaged phagemids can convert the single stranded DNA to double stranded DNA and maintain it as a plasmid.

For each lambda ZAP phage stock ($>10^5$ pfu/ml) generated from a plaque that positively hybridized, 200 µl was mixed with 1 µl of helper phage R408 (1×10^{12} pfu/ml) and added to 200 µl of XL1-Blue cells ($O.D._{600}=1.0$) in a sterile tube. The tube was incubated at 37°C for 15 minutes and 5 ml of 2X YT medium (see Appendix) was added. The mixture was incubated at 37°C for 3 hours with shaking. The samples were then heated at 70°C for 20 minutes to kill the *E. coli* host cells, and centrifuged in a clinical centrifuge for 10 minutes. The supernatant, containing single stranded pBluescript phagemids packaged in f1 phage particles, was transferred to a fresh sterile tube. To produce double stranded plasmid 200 µl of the phage stock was added to 200 µl of an overnight culture of XL1-Blue cells in a small plastic tube. The tube was incubated at 37°C for 15 minutes, and 1 to 100 µl aliquots were plated onto LB plates containing ampicillin (see Appendix). The plates were incubated overnight at 37°C. Resulting colonies were used to innoculate 5 ml LB tubes containing ampicillin, and the tubes were incubated overnight at 37°C with shaking. The recombinant plasmids (consisting of *cya-5*$^+$ cDNA inserts in pBluescript) were isolated from the cultures by a rapid plasmid DNA isolation procedure (page 36), and characterized by digestion with various restriction enzymes followed by gel electrophoresis.

Cloning of cya-5$^+$ cDNA by the RACE procedure

In an attempt to clone the 5' end of the *cya-5*$^+$ cDNA, the RACE (Rapid Amplification of cDNA Ends; Frohman et al., 1988) protocol was followed with minor modifications. The first strand synthesis of cDNA was carried out as follows. Initially, 20 pmol (1 µl) of 5' RT primer (Fig. 11) was added to 1 µg (3 µl) of poly A RNA isolated from wild-type strain NCN10 in a sterile eppendorf tube. Sterile, distilled water was then added to a final volume of 8 µl, and the mixture was heated at 70°C for 10 minutes. The mixture was chilled on ice for 1 minute, and centrifuged briefly. This was followed by the addition of 1 µl of 1 U/µl RNase Block II (an RNase inhibitor;

Stratagene), 4 µl of 5X first strand reaction buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 µl of 0.1 M dithiothreitol, 1 µl of a mix consisting of each dNTP at a concentration of 10mM, and 30 µCi (3 µl) of [alpha-^{32}P]dCTP. The mixture was incubated at 37°C for 2 minutes to equilibrate the temperature, and 200 units (1 µl) of MMLV reverse transcriptase was added. The reaction mixture was incubated at 37°C for 1 hour.

Excess 5' RT primer was removed from the reaction mixture by spin column chromatography through a Sephadex G-50 column (Maniatis et al., 1982). The single stranded DNA and RNA collected from the column was adjusted to a volume of 50 µl with sterile, distilled water, and precipitated with one-tenth volume of 3 M sodium acetate (pH 7.5) and 3 volumes of 95% ethanol. The DNA and RNA pellet was resuspended in 22 µl of sterile, distilled water. For tailing of the 3' end of the first strand cDNA, 1 µl of 6 mM dATP, 6 µl of 5X tailing buffer (Bethesda Research Laboratories) and 10 units (1 µl) of Terminal deoxynucleotidyl transferase (Bethesda Research Laboratories) were added to the mixture. The reaction was allowed to proceed at 37°C for 10 minutes and was then terminated by incubating at 65°C for 15 minutes. The mixture was diluted to 500 µl in TE buffer, 5 µl of which was used in PCR.

The reaction mixture of the first round of amplification by PCR consisted of the cDNA pool (5 µl), 10 pmol (1 µl) of (dT)17-adaptor primer, 25 pmol (1 µl) of the adaptor primer, and 25 pmol (1 µl) of the 5' RT primer used for reverse transcription (Fig. 11). To this mixture 5 µl of 10X Vent DNA polymerase buffer (1X buffer is 10 mM KCl, 10 mM ammonium sulfate, 20 mM Tris-HCl pH 8.8, 2 mM MgSO4, 0.1% Triton X-100), 2.5 µl of 2 mg/ml of BSA, 7.5 µl of 10 mM dNTP mix (final concentration of each dNTP being 1.5 mM; see Appendix), 3.4 µl of 100 mM MgSO4 (final concentration of 6.8 mM) and 1 µl of Vent DNA polymerase (New England Biolabs) were added and thoroughly mixed. The reaction was overlaid with mineral oil and 30 cycles of PCR were carried out in a Robocycler 40 temperature cycler (Stratagene) as follows. The first cycle of PCR consisted of denaturation at 94°C for 5 minutes, annealing at 45°C for 2 minutes and primer extension at 72°C for 5 minutes. The subsequent 29 cycles consisted of: 93°C, 1 minute; 45°C, 2 minutes; 72°C, 2 minutes. A second round of PCR was then carried out with 1 µl of the first round product. Reaction conditions in the second round of amplification differed in that the (dT)17-adaptor primer was omitted, and 25 pmol of 5' amp primer (Fig. 11) was used in lieu of the 5' RT primer used in the first round. The adaptor primer (25 pmol) was used as in the first round of amplification. All other reaction constituents were also identical to the first round of PCR. The first cycle of PCR consisted of: 94°C, 5 minutes; 62°C, 2 minutes; 72°C, 2 minutes. This was followed by 29 cycles of: 94°C, 1 minute; 62°C, 2 minutes; 72°C, 2 minutes. A fraction of the first and second round PCR products were then characterized by agarose gel electrophoresis and southern analysis.

Restriction analysis and agarose gel electrophoresis of DNA

Restriction endonucleases and the appropriate buffers were purchased from Bethesda Research Laboratories (BRL), Inc., New England BioLabs, Inc., or Boehringer Mannheim. Reactions consisted of the DNA, the appropriate amount of buffer (purchased in 5X or 10X concentrations), distilled water and 10 to 20 units of enzyme. Reactions were carried out at 37°C for 1 to 2 hours.

Restriction fragments were electrophoresed on 0.8% (w/v) agarose gels in 0.1 M Tris-borate pH 8.3, 2 mM EDTA, containing ethidium bromide (0.5 µg/ml). DNA samples were made to approximately 5% (v/v) glycerol before loading on a gel. Electrophoresis was carried out at a constant voltage of 25 to 100 volts.

Purification of DNA from agarose gels

Fragments of DNA were purified from gels using a Geneclean kit provided by BIO 101 Inc. Agarose slices containing the DNA of interest were weighed, cut into small pieces, and placed in an eppendorf tube. Approximately 2.5 to 3 volumes of 6 M sodium iodide was added to the agarose, and the tube was incubated at 45°C to 55°C for 5 minutes to melt the agarose. This was followed by the addition of 5 µl of Glassmilk (a suspension of silica matrix in water), which binds DNA. The tube was mixed thoroughly and incubated on ice for at least 10 minutes, mixing the contents of the tube every 2 minutes to ensure that the Glassmilk stays suspended. The DNA bound silica matrix was pelleted in a microcentrifuge for 5 seconds. The pellet was washed 3 times with NEW solution (containing NaCl, ethanol and water and provided in the Geneclean kit) by alternately resuspending and pelleting the Glassmilk. The DNA was then eluted from the Glassmilk by resuspending in 10 µl of distilled water and incubating at 45°C to 55°C for 2-3 minutes. The tube was centrifuged for 30 seconds, and the DNA-containing supernatant was transferred to a new tube. Any remaining DNA still bound to the Glassmilk was eluted with another 10 µl of water by repeating the above. The eluates were combined, and a small amount of the purified DNA was electrophoresed on an agarose gel to estimate the yield.

Mitochondrial RNA isolation

Mitochondria were isolated from sucrose gradients by the method of Lambowitz (1979). All steps were carried out at 4°C. Mycelia from 2 litre cultures of NCN5 and NCN10 strains (grown for 30 hours and 16 hours, respectively) were harvested by filteration. The mycelia were ground in a mortar in a solution of 15% sucrose, 10 mM Tris-Cl (pH 7.5), 0.2 mM EDTA and centrifuged in a Sorvall GSA rotor (2500 rpm, 10 minutes) to pellet nuclei and cell debris. The supernatant was centrifuged again in a GSA rotor (10 000 rpm, 30 minutes) to pellet mitochondria. The pellet was resuspended in 5-10 ml of 20% sucrose, 10 mM Tris-Cl (pH 7.5), 0.1 mM EDTA and the mitochondria were pelleted at 10 000 rpm for 30 minutes. The supernatant was decanted, taking care to remove excess 20% sucrose, and the pellet was carefully resuspended in 5 ml of 60% sucrose, 10 mM Tris-Cl (pH 7.5), 0.1 mM EDTA. A step gradient was then set up in SW 41 tubes. The mitochondrial suspension was transferred to SW 41 tubes (no more than 0.5 ml of mitochondrial suspension per tube), and was then carefully overlaid with 4 ml of 55% sucrose solution (55% sucrose, 10mM Tris-Cl (pH 7.5), 0.1 mM EDTA) followed by 3ml of 44% sucrose solution (44% sucrose, 10 mM Tris-Cl (pH 7.5), 0.1 mM EDTA). The tubes were centrifuged in a SW 41 rotor at 40 000 rpm for 90 minutes. The mitochondria form a tight band at the interface of the 44% and 55% sucrose layers. The mitochondria were removed with a Pasteur pipette into a sterile tube.

Mitochondrial RNA was isolated from purified mitochondria by a method described by Breitenberger *et al.* (1985). Mitochondria obtained from a sucrose gradient were resuspended in 2 ml of 10 mM Tris-HCl (pH 7.6), 10 mM CaCl2, 100 mM NaCl, 1 mM EDTA and 1% SDS. Then DEPC (0.005%) was added to inhibit ribonucleases. The mixture was extracted with 2 ml of phenol, followed by 2 ml of chloroform. The RNA was then precipitated by the addition of ammonium acetate to a final concentration of 2.5 M, followed by the addition of 3 volumes of 95% ethanol. The RNA was allowed to precipitate overnight at -20°C, and was then centrifuged in an SS34 rotor at 10 000 rpm for 1 hour. The RNA was redissolved in 50 μl of TE buffer, and treated to remove contaminating DNA as follows. A mixture containing (per sample) 10 μl of 100 mM $MgCl_2$/10mM dithiothreitol solution, 0.4 μl of 10 U/μl RNase-free DNase, 0.2 μl of

1U/ul RNase Block II (Stratagene; an RNase inhibitor), and 39.4 µl of TE buffer was prepared, and 50µl of this mixture was added to each RNA sample. The reaction was incubated at 37°C for 15 minutes. The reaction was stopped by adding 25 µl of DNase stop mix (50 mM EDTA, 1.5 M sodium acetate, 1% SDS) to each sample. Each sample was then extracted once with phenol/chloroform/isoamyl alcohol (24:24:1) and once with chloroform/isoamyl alcohol (24:1). The RNA was then precipitated by adding 325 µl 95% ethanol and incubating for 30 minutes to overnight at -20°C. The RNA was pelleted by centrifugation (15 minutes, 4°C), and washed with 70% ethanol. The pellet was dried and resuspended in 100 µl of water. The RNA was electrophoresed on formaldehyde-containing 1% agarose gels for Northern analysis.

Labelling of mitochondrial translation products

Mitochondrial translation products were labelled and isolated by the procedure of Bertrand and Werner (1977). Cultures of NCN5, NCN10 and NCN80 were incubated at 30°C in 500 ml of liquid Vogel's medium (see Appendix), which had been innoculated with 1 X 10^9 conidia from each strain. The NCN5 and NCN80 cultures were grown for 35 hours and the NCN10 culture was grown for 16 hours, which was the approximate time for the cultures to reach log phase. To 100 ml of each exponentially growing culture, 10 mg of cycloheximide (final concentration of 0.1 mg/ml) was added and mixed for 2.5 minutes before the addition of 1 mCi of L-[4,5-^{3}H]leucine. Each culture was then incubated for 1 hour at 30°C with shaking to allow incorporation of the label into mitochondrial translation products. The cultures were harvested by vacuum filtration in a Buchner funnel, washed with ice cold distilled water, and frozen immediately with liquid nitrogen. All subsequent steps were performed at 4°C. Grinding buffer (500 µl of 15% sucrose, 50 mM Tris-HCl, pH 7.8 at 4°C, 0.25 mM EDTA), 5 µl of PMSF (200 mM in ethanol or ethyl acetate) and a small amount of acid-washed sand were added to the frozen mycelium, which was then ground to a paste in a mortar. The mixture was then suspended in grinding buffer, and centrifuged (3000 rpm for 10 minutes) in a SS-34 rotor to pellet the debris. The supernatant was decanted to a new tube, and the mitochondria were pelleted (12 000 rpm for 25 minutes) in a SS-34 rotor. The supernatant was discarded and the mitochondria were washed by resuspending in about 1 ml of grinding buffer containing PMSF. The mitochondria were pelleted (12 000 rpm, 25 minutes) in a SS-34 rotor, and resuspended in a 100 µl of 0.1 M sodium phosphate (pH 8.0 at 4°C). A small amount (5µl) of the mitochondrial suspension was counted in a scintillation counter. The suspension was stored at -20°C, and 100 000 cpm were loaded on a gel.

Polyacrylamide gel electrophoresis of proteins.

Mitochondrial translation products were electrophoresed on Laemmli (1970) gels. Gels were made up of a stacking gel and a resolving gel (see Appendix). The running buffer consisted of 25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS. Proteins were denatured by the addition of an equal volume of 5% SDS, 0.0625 M Tris-HCl (pH

6.8), 5% (v/v) ß-mercaptoethanol, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue. The samples were left at room temperature for 3-4 hours, and centrifuged for 5 minutes in a microcentrifuge before loading the supernatant. The samples were electrophoresed at 80 volts (constant voltage) overnight until the bromophenol blue had migrated at least 10 cm into the resolving gel. The gel was immersed in destain solution (water, methanol and acetic acid in a ratio of 5 : 4 : 1) overnight to remove SDS. The gel was stained (in a solution identical to destain solution except it also contains 0.25% coomassie brilliant blue) for at least 5 hours, and destained for 5-8 hours. The gel was then placed in Amplify fluorographic reagent (Amersham) for 1 hour, and dried at 60°C for 4 hours in a Slab Drier (BioRad) model 483. The gel was autoradiographed at -70° C .

Results and Discussion

COX I is not detected in mitochondrial translation products of the *cya-5* mutant.

The deficiency of subunit I of cytochrome *c* oxidase (COX I) in the *cya-5-34* mutant was demonstrated by directly labelling mutant mitochondrial translation products *in vivo.* Mutant and wild-type cultures were labelled with ^{3}H-leucine in the presence of cycloheximide (which inhibits cytosolic translation, but not mitochondrial translation), and mitochondria were isolated. Mitochondrial translation products were electrophoresed on a SDS-polyacrylamide gel (Laemmli et al., 1970), which was dried and autoradiographed (Fig. 3). Comparison of mitochondrial translation products in two *cya-5* strains (NCN5 and NCN80) and a wildtype strain (NCN10) reveal an apparently complete deficiency of COX I in the mutants. These results confirm previous findings (Nargang et al., 1978) that *cya-5* is deficient in COX I. Since cytochrome oxidase subunit 2 (COX II) and cytochrome *b* (COB) were not resolved on the gel shown (appear as one band), the previously reported reduced levels of COX II (Nargang et al., 1978) in the mutant was not confirmed. However, subunit 3 of cyochrome oxidase (COX III) was detected at approximately wild-type levels (Fig. 3).

Analysis of cytochrome *c* oxidase in *cya-5* was previously done by immunoprecipitating subunits with appropriate antibodies (Nargang et al., 1978; see Introduction). The polypeptides were also analyzed by *in vivo* labelling of mitochondrial translation products, as in this study. However, in the previous work, the polypeptides were detected by determination of the total radioactive counts in gel slices. COX 1 was not detected in *cya-5* by that method, nor by autoradiography in this study, suggesting that COX 1 is absent or present below the level of detection of both methods.

Sequencing of a genomic clone that rescues the *cya-5* mutant phenotype

Genetic analysis has shown that the *cya-5* phenotype is caused by a mutation in a single nuclear gene (Nargang et al., 1978). The wild-type gene was previously cloned by Dr. John Coffin in this laboratory by mutant rescue using the sib selection procedure described by Akins and Lambowitz (1985). Using this procedure, a single cosmid clone carrying the *cya-5*$^+$ gene was isolated from a wild-type *N. crassa* (strain 74A) genomic cosmid library. This library contains *N. crassa* genomic DNA cloned in the vector pSV50 (Fig. 4). In order to identify the *cya-5*$^+$ gene by DNA sequence analysis, the cosmid clone was subcloned into the vector pUC19 as a *ca*. 6.4 kilobase Sph1-Xma1 fragment (plasmid pJC2; Table 4) that rescued the *cya-5* mutant. The complete DNA sequence, from both strands, was determined from the Ssp1 restriction endonuclease site to position 6000 by previous workers (Fig. 5). The sequence of the region between nucleotide 6000 and XmaI (a total of approximately 400 bp) was not determined, because transformation experiments (see below) suggested that the region between SnaBI and XmaI did not lie inside the *cya-5*$^{+}$ gene.

In the present study, the remainder of the pJC2 insert extending from the Ssp1 site to the Sph1 site (a total of 1586 bp) was sequenced on both strands (Fig. 5). The entire sequenced region was examined in all six reading frames present on both strands for uninterrupted ORFs of 50 amino acids or greater (Fig. 5). This region contains many large ORFs potentially coding for the putative *CYA-5* protein, the most striking of which is 1136 amino acids long. Although it seems likely that an ORF of this size would code for a protein, without further analysis, none of the ORFs can be ruled out as potentially coding for the *CYA-5* protein. If the *cya-5*$^{+}$ gene contains one or more introns, it is possible that more than one of the ORFs could code for the protein. Also to be considered is the possibility that one or more ORFs could represent other genes closely linked to *cya-5*$^{+}$.

Determination of the limits of the *cya-5*$^{+}$ gene by restriction enzyme/transformation analysis

Because of the relatively large size of the *cya-5*$^{+}$ subcloned fragment (6.4 kb SphI-XmaI clone) and the many ORFs present therein, it was deemed beneficial to obtain the approximate limits of the *cya-5*$^{+}$ gene, in order to determine which ORFs most likely encoded the protein. This was achieved by digesting the plasmid pJC2 (Table 4) with several restriction enzymes which have single recognition sites in the subcloned fragment, and examining whether this DNA could rescue the slow growth character of the *cya-5* mutant upon transformation. Efficient rescue of the mutant would indicate that an enzyme recognition site lies outside the gene, whereas failure to do so would suggest that a site is within the gene.

The plasmid pJC2 was digested with either EcoRI, KpnI, SnaBI, SspI or XmaI (Fig. 5), and co-transformed with undigested pSV50 (Fig. 4) DNA into *cya-5* sphaeroplasts (Fig. 6). Co-transformation was done because pSV50 provides the gene for benomyl resistance (Fig. 4), thus allowing selection for transformants on media containing benomyl. This helps reduce the background of slow-growing colonies that would be obtained if rescue were singly of the *cya-5* phenotype. Rapidly growing colonies appearing on benomyl plates were judged to be *cya-5*$^+$/Ben^R transformants. Table 5 summarizes the results of one set of experiments. Transformations with EcoRI, KpnI and SspI digested DNA resulted in very few or no fast growing transformants. This suggests that digestion of pJC2 with these enzymes destroys the *cya-5*$^+$ gene, and, therefore, these restriction sites are within the gene. This data supports the notion that the 1136 amino acid ORF encodes the *CYA-5* protein, for it is the only contiguous ORF that encompasses all three restriction sites (Fig. 5). The result of the transformation with SnaBI digested DNA is ambiguous (Table 5). The relatively large number of rescued transformants in comparisons with the negative control in which pSV50 is transformed alone, and with EcoRI, KpnI and SspI digested pJC2, suggest that the SnaBI site is outside the gene. This is consistent with the possibility that the 1136 amino acid ORF encodes the *CYA-5* protein, since the SnaBI site is not within this ORF (Fig. 5). However, the number of rescued transformants with SnaBI digested DNA is still significantly less than that observed with the undigested plasmid (pJC2 and pSV50). This difference can be attributed in part to what appears to be differences in transformation efficiency between circular and linearized plasmid DNA. That is, plasmid digested with XmaI, which has an intact *cya-5*$^+$ gene, since XmaI is one of the cloning sites of pJC2, also produced a significantly lesser number of transformants than undigested plasmid (Table 5). Nevertheless, SnaBI digested DNA still appears to rescue the *cya-5* strain relatively inefficiently (compare values obtained from XmaI and SnaBI digested DNA, Table 5).

The restriction enzyme/transformation data is most consistent with the possibility that at least most of the 1136 amino acid ORF encodes the *CYA-5* protein. However, other non-contiguous ORFs cannot be ruled out, since possible introns in the gene have not been defined.

Obtaining cDNA clones of *cya-5*$^+$

In order to identify the 5' and 3' ends of the *cya-5*$^+$ gene, as well as possible introns, two wild-type *N. crassa* cDNA libraries were screened for *cya-5*$^+$ cDNA clones. The libraries were constructed in bacteriophage lambda ZAP (Stratagene), and were generously provided by Dr. Matthew Sachs (Stanford University , Stanford, California). One library had been constructed from poly A RNA isolated from mycelial culture, while the second library was constructed from poly A RNA isolated from a culture of germinating conidia. Approximately 800 000 lambda ZAP clones were plated in total from both libraries, and plaque lifts were performed. The filters were hybridized with the SphI-XmaI fragment of pJC2 containing the *cya-5*$^+$ gene (Fig. 5). In total, six clones were identified from both libraries (2 from the mycelial library and 4 from the conidial library) that gave positive signals in hybridization reactions. By the automatic excision protocol (Fig. 2), phagemids (containing a functional bacteriophage f1 origin of replication) were generated that consisted of the cDNAs cloned in the vector Bluescript (plasmid pRDC1; Table 4). Restriction enzyme and sequence analysis revealed that all 6 independently isolated clones were identical. They were 2747 bp long and corresponded to most of the 1136 amino acid ORF (Fig. 7). No other clones were isolated, adding further support to the contention that the 1136 amino acid ORF encodes the *CYA-5* protein, and that no other mRNAs are encoded from the ~6.4 kb SphI-XmaI fragment (Fig. 5).

The cDNA was completely sequenced, and was discovered to be identical to the genomic sequence, thus revealing no introns (Fig. 8). The 3' end of the gene was identified at position 4987, but the cDNA was incomplete at the 5' end, terminating at the EcoRI site (position 2240; Figs. 7 and 8). Once these results were obtained, it was discovered that incomplete cDNA clones (of other genes) that terminate at internal EcoRI sites have previously been isolated from the two libraries used in this study (Dr. M. Sachs, personal communication). The libraries were constructed by cloning into the EcoRI site of the lambda ZAP vector. The most likely explanation for the above observations, therefore, is that there was incomplete methylation of cDNAs by EcoRI methylase during construction of the library. This resulted in digestion of some cDNAs at unprotected EcoRI sites during cloning.

Cloning of the 5' end of the *cya-5*$^+$ cDNA

An attempt was made to obtain the 5' end of the *cya-5*$^+$ transcript by the RACE (Rapid Amplification of cDNA Ends) procedure (Frohman et al., 1988; Fig. 9). In this technique, cDNAs are generated by using the polymerase chain reaction (PCR) to amplify a region between an internal site of a transcript and its 3' or 5' end. The minimum information required is a single stretch of sequence within the mRNA to be cloned, which is used as a gene specific primer.

In order to clone the 5' end of the *cya-5*$^+$ gene, a gene specific primer (5' RT) homologous to a sequence 164 nucleotides downstream of the EcoRI site (Fig. 8) was chosen for reverse transcription of a wild-type poly A RNA preparation (Fig. 9). After removing excess 5' RT primer and adding a poly A tail to the cDNA with terminal deoxynucleotidyl transferase, the first strand reaction product was amplified by PCR. There were 3 primers used in the amplification (Figs. 9 and 10): the 5' RT primer, an adaptor primer (which places a unique sequence at the unknown end of the cDNA), and a primer consisting of the adaptor sequence upstream of 17 dT residues (dT_{17}-adaptor primer). The amplification reaction involves annealing of the $(dT)_{17}$-adaptor primer to the poly A tail synthesized on the first strand cDNA, and synthesis of a complementary second strand. The adaptor primer and the gene specific 5' RT primer then amplify the cDNAs in subsequent PCR cycles. However, the reaction tends to be inefficient; non-specific products are often amplified, in addition to the product of interest (Frohman et al., 1988). Specificity of the reaction largely depends on the 5' RT primer only hybridizing to the mRNA of interest. Therefore, it is often necessary to perform a second round of amplification using the adaptor primer and a nested gene specific primer (5' amp) homologous to a site upstream of the 5' RT sequence. This adds further specificity to the amplification, since any non-specific products generated from the first round of PCR should not be amplified further.

Following the procedure outlined above, a specific product (865 bp) was amplified that was detectable on an ethidium bromide stained gel, and in southerns using the SphI-EcoRI fragment of pJC2 as a probe (Fig. 11A). As often observed in the procedure (Frohman et al., 1988), some conditions produced no detectable product after one round of PCR amplification (in ethidium bromide stained gels or in southerns), but a product was visible after a second round of PCR was performed using an internal nested (5' amp) primer (Fig. 10) to increase specificity of the reaction (Fig. 11A, lanes b, d and f). This product was cloned into the vector pBS (plasmid pRDC2; Table 4) and sequenced to identify the 5' end of the *cya-5*$^+$ transcript. According to the procedure, the sequence of the 5' end should be preceeded by a string of T residues corresponding to the poly A tail that was added onto the 5' end of the first cDNA strand synthesized by reverse transcription (Fig. 9). This string of T residues should in turn be preceeded by sequence from the adaptor primer (Fig. 9). However, only the adaptor primer sequence preceeded sequence from the *cya-5*$^+$ gene. No T residues were observed between these sequences, suggesting that the adaptor primer had annealed at a different site in the *cya-5*$^+$ cDNA (not at the 5' end). Examination of the sequence that had been amplified revealed that the adaptor primer had in fact annealed to a site (at positions 1451-1473 of the sequence shown in Fig. 8) that contained 5 mismatches out of 22 nucleotides from the adaptor sequence (and was, presumably, downstream of the actual 5' end of the *cya-5*$^+$ transcript). The entire truncated RACE product was 865 bp long and matched the genomic sequence; therefore, no introns were discovered (see Figs. 8 and 10 for location of 5' amp and 5' RT primers, and the site of mispriming of the adaptor primer).

The amplification reactions described above were performed at a low annealing temperature of 45°C. The PCR reactions were repeated at higher annealing temperatures in an attempt to increase the stringency of the reaction to favour annealing of the adaptor primer to the 5' end of the *cya-5*$^+$ transcript, instead of the homologous downstream site. Annealing temperatures of 50°C, 55°C, and 62°C were tested. Surprisingly and unfortunately, bands of the same size as the truncated RACE product were detected at all temperatures in ethidium bromide stained gels (Fig. 11 B). Higher annealing temperatures, therefore, did not allow amplification of a larger product. Attempts at PCR using low annealing temperatures (42°C or 45°C) for the first 5 cycles of the first round of amplification, followed by 25 cycles of higher annealing temperatures (50°C, 55°C or 60°C) were also made. This was done to examine the possibility that higher annealing temperatures in the first few cycles of PCR were preventing annealing of the poly T tail of the (dT)17-adaptor primer to the A residues synthesized onto the 5' end of the first strand *cya-5*$^+$ cDNA. These conditions also did not amplify a larger product than the one previously detected (data not shown). We are currently attempting to characterize the 5' end of the *cya-5*$^+$ gene by using a different adaptor primer for the RACE procedure, and by screening different cDNA libraries.

Although the complete 5' end of the *cya-5*$^+$ cDNA was not obtained using the RACE procedure, an additional 766 bp was identified upstream of the sequence obtained from the cDNAs isolated from the lambda ZAP library (Figs. 7 and 8). However, it is possible that the RACE product is not *cya-5*$^+$ cDNA, but is the result of amplification of contaminating genomic DNA present in the original poly A RNA preparation that was used for the RACE procedure. To test the possibility that there is contaminating genomic DNA present in the poly A RNA, a pair of primers (THA 1 and 2) designed to amplify sequences from another gene (*N. crassa MOM 19* gene; Schneider et al., 1991) were used in PCR reactions of the RACE preparation of poly A RNA and single stranded *cya-5*$^+$ cDNA (generated from reverse transcription). These primers do not share significant homology with sequences in the SphI-XmaI restriction fragment containing the *cya-5*$^+$ gene. Since the only single-stranded cDNA that should be present in the mixture used as a template is *cya-5*$^+$ cDNA, no amplification product is expected with the THA 1 and 2 primers, unless there is genomic DNA present in the preparation of poly A RNA (in which case a 1297 bp product should be detected). No product was detected with these primers under various conditions (Fig. 12, lanes d-f). As a positive control, two primers (5' amp and RDH22) that consisted of sequences that were a part of the previously identified RACE product (Figs. 8 and 10) were also tested. The expected 542 bp product was detected in southerns (Fig. 12, lane g). The results suggest that there is no DNA contamination in the poly A RNA which might account for the truncated RACE product described above. Thus, it seems likely that this product is amplified *cya-5*$^+$ cDNA.

Interestingly, when two additional primers, RDH5 and RDH1, derived from sequences at positions 551-567 and 1244-1261, respectively (Figs. 8 and 10), were tested in PCR reactions of the RACE mixture of poly A RNA and first strand *cya-5*$^+$ cDNA, a product was amplified that was of the size expected (710 bp) if the *cya-5*$^+$ transcript extends at least as far upstream as the RDH5 primer (Fig. 12, lanes A-C). This site is 884 nucleotides upstream of the truncated RACE product (Fig. 8).

Analysis of the DNA sequence

The DNA sequence of the SphI-XmaI restriction fragment has been examined for similarity to the consensus sequence established for *N. crassa* translation initiation sites (Ballance, 1986; Gurr et al., 1987). This sequence comprises the first 8 nucleotides immediately preceeding the ATG initiation codon, and may play a role in targeting the ribosome to the correct start site (Kozak, 1989). Sequences upstream of several ATGs show some similarity to the consensus sequence. The sequence preceeding the first methionine codon of the 1136 amino acid ORF (position 1289, Fig. 8), in particular, displays a high degree of similarity to the consensus sequence in comparison to other sequences. This sequence matches the consensus sequence at 6 out of the 8 positions (Fig. 13). Furthermore, this sequence contains a G residue at position -3 (relative to the ATG codon), which is a position highly conserved for purines among translation initiation sites in eukaryotes (Kozak, 1989), including those of *N. crassa* (all *N. crassa* genes listed in Fig. 13 have an A or G at this position). Also noteworthy is the C residue at position -4 (Fig. 13) which is strongly conserved in *N. crassa* genes. Support for this methionine being the *cya-5*$^+$ translation initiation site comes from two other observations. Since the *cya-5*$^+$ gene product appears to effect the expression of subunit one of cytochrome *c* oxidase, it seems reasonable that the protein should contain a targeting signal for import into mitochondria. A targeting sequence is most often found at the amino terminus of mitochondrial proteins (Hartl et al., 1989). By performing a computer search using the rules set forth by Gavel and von Heijne (1990), a targeting peptide was predicted only 5 amino acids downstream of the putative translation start site (Fig. 8). This was the only predicted targeting peptide in the entire *Sph*I-*Xma*I region. It is rich in hydrophobic and basic amino acids, characteristic of mitochondrial targeting peptides (Hartl et al., 1989).

Further support for the methionine at position 1289 being the *CYA-5* translation start site comes from examination of translation initiation in other eukaryotes. In the vast majority of cases (>90%) in eukaryotes, including *N. crassa*, translation begins at the proximal methionine of a protein coding reading frame (Kozak, 1983;1989). In many cases, methionine codons exist in the untranslated leader sequence of mRNAs upstream of the translation start site, but these are almost always followed by in frame stop codons shortly downstream (and, therefore, are not part of the protein coding reading frame). In fact, it has been demonstrated that short ORFs in the untranslated leader of mRNAs can play an inhibitory role in translation (Kozak, 1989). Nevertheless, Kozak's Scanning model of translation initiation (Kozak, 1989) predicts that translation initiation sites are selected on the basis of favourable sequence contexts and position (with the 5' most ATG in the reading frame being favoured). The methionine at position 1289 meets both conditions of this model. It is in the best agreement with the established consensus sequence, and it is the first methionine codon in the 1136 amino acid ORF, only 37 nucleotides downstream from an in frame termination codon (Fig. 8).

The *cya-5*$^+$ cDNA sequence has not been defined as far upstream as the putative translation initiation site. The 5' end of the misprimed RACE product is 185 nucleotides downstream of this site (Fig. 8). Based on analysis of the genomic sequence, there appears to be no introns in this 185 nucleotide region, as there is no similarity to well conserved consensus sequences characterizing *Neurospora* introns (Gurr et al., 1987). Since there are also no introns in the rest of the gene for which cDNA sequence has been obtained (see above), the predicted size of the *CYA-5* protein is 1136 amino acids, the same size as the ORF defined by sequencing the genomic clone (Figs. 7 and 8). The protein has a predicted molecular weight of 130 029 Daltons.

A search for sequences characteristic of eukaryotic promoter elements, such as TATA boxes and CAAT boxes (Montague, 1987), revealed no obvious similarities upstream of the putative translation initiation site (although the transcription initiation site would have to be known for such a search to be meaningful). However, many fungal genes lack such elements (Gurr et al., 1987). For those fungal genes in which such sequences have been observed, the similarity to the canonical sequences is often weak, and their role in gene expression is uncertain (Gurr et al., 1987).

However, in the 3' untranslated region sequences typical of poly A signalling sites (thought to be involved in polyadenylation of the 3' termini of mature mRNA) are found (Gurr et al., 1987). A sequence exactly matching the consensus sequence, AUAAA, is present 18 nucleotides downstream of the termination codon (Fig. 8). A similar sequence, AUAA, is found 85 nucleotides downstream of the stop codon.

Codon usage within the *cya-5*$^+$ gene has been examined (Table 6). It is similar to other *Neurospora* genes in that where there is a choice, C in the third position of codons is favoured, and there is a bias against A in this position. However, highly expressed genes in *Neurospora,* such as *am* or *tub-2*, exhibit much stronger bias against A in the third position than do weakly expressed genes, such as *trp-1* (Gurr et al., 1987). To illustrate, only 0.44% of codons in *am* and 1.12% of codons in *tub-2* end in A, whereas 53.7% and 54.9% of codons end in C, respectively. In *trp-1* the bias is not as great with 5.39% and 45.3% of codons ending in A and C, respectively. The *cya-5*$^+$ gene resembles weakly expressed genes like *trp-1*, with 13.8% of codons ending in A, and 34.8% of codons ending in C. In fact, the bias against A appears to be weaker than even *trp-1*, suggesting that the *CYA-5* protein may be expressed at extremely low levels. This is consistent with the results of Northern analysis, in which the *cya-5*$^+$ transcript was undetectable (see below).

A homology search of the *CYA-5* protein sequence was done against all proteins in the Swiss prot data base (release 24) and EMBL data base (release 33), and no significant matches were found. Sequence alignments were carried out between *CYA-5* and various nuclear encoded yeast proteins that are required for expression of mitochondrial genes (*PET494, PET111, CBP1, CBP2, CBS1, CBS2, CBP6,* *PET54*, *PET122* and *PET123*), since *CYA-5* likely carries out a related function (see below). This analysis revealed no striking similarity with any of the proteins. However, there is no significant homology among any of the yeast proteins, either (Grivell, 1989).

Northern analysis of the *cya-5*$^+$ transcript

Codon usage analysis suggested that *cya-5*$^+$ is expressed at a low level. Northern analysis was performed in an attempt to detect the transcript in RNA preparations from the *cya-5* mutant, wildtype *74A* and two other unrelated mutant strains (the *[mi-3]* extranuclear mutant and the *cyt-2-1* mutant; see Materials and Methods for description of mutants). Poly A and total RNA preparations of these strains separated on formaldehyde gels were blotted onto nylon membranes and hybridized with probes derived from the *cya-5*$^+$, *cyt-2*$^+$ and *tub-2*$^+$ genes (see legend of Fig. 14 for description of probes). The *cyt-2*$^+$ gene, which codes for cytochrome *c* heme lyase, serves as a good control, because it has previously been shown to be expressed at low levels, as predicted for the *cya-5*$^+$ gene (Drygas et al., 1989). The constitutively expressed *tub-2*$^+$ gene, coding for ß-tubulin (Orbach et al., 1986), was also used as a control. It serves as an example of a highly expressed gene for comparison.

As illustrated in Figure 14, the *cya-5*$^+$ transcript was not detectable in any of the RNA preparations after six days exposure of Northern blots. Longer exposures of blots that were hybridized with a *cya-5*$^+$ probe of higher specific activity (see legend, Fig. 14), still did not give a signal. However, the *cyt-2*$^+$ transcript was detected in the *cya-5* mutant total RNA and all poly A RNA preparations after exposure for the same amount of time, and hybridization of a probe with similar specific activity (Fig. 14). Furthermore, *cyt-2*$^+$ expression appears to be induced in the *cya-5* mutant above levels present in the wildtype strain (Fig. 14, *cyt-2* probe, lanes d and e); a *cyt-2*$^+$ signal was detected in total RNA from the former, but not the latter, despite there being approximately equal amounts of both RNAs as judged by the intensities of the *tub-2* bands (Fig. 14, *tub-2* probe, lanes d and e).

The results of Northern analysis indicate that the *cya-5*$^+$ gene is expressed at very low levels, consistent with codon usage analysis, which predicts that the *CYA-5* protein is expressed at low levels. Conditions which allow for the detection of *cyt-2*$^+$ message, a gene known to be expressed at low levels (Drygas et al., 1989; compare signals from *cyt-2* and *tub-2* probes, Fig. 14), did not allow detection of *cya-5*$^+$ message. That *cyt-2*$^+$ may be induced in *cya-5* mutant cultures is not surprising considering the initial finding that *cya-5* has a 2-fold greater amount of spectrally detectable cytochrome *c* than wildtype strains (Nargang et al., 1978). The *cyt-2*$^+$ gene product is required for the addition of the heme prosthetic group to cytochrome *c* (Drygas et al., 1989). Where there is an increase in active cytochrome *c*, one might expect a corresponding increase in *cyt-2*$^+$ expression.

Detection of the COX I transcript in the *cya-5* mutant

It was previously shown (Nargang et al., 1978), as well as in this study (see above), that *cya-5* is specifically deficient in mitochondrially encoded subunit one of cytochrome *c* oxidase. In order to gain further insight into the function of the *cya-5*$^+$ gene product, it was of interest to determine whether or not the COX I transcript is detectable in the mutant. Northern blots of mitochondrial RNA prepared from wild-type and mutant strains hybridized with 3 different mitochondrial gene probes (derived from the COX I, COX III and COB genes) is shown in figure 15. The mature, approximately 5 kb COX I transcript is clearly detectable in all lanes corresponding to *cya-5* mitochondrial RNA (Fig. 15 B, lanes b-e). Moreover, comparison of lanes a (0.5 μg of wild-type mitochondrial RNA) and c (0.4 μg of mitochondrial RNA from *cya-5*) suggests that the COX I transcript may be present at higher levels in *cya-5* than in the wildtype strain. The approximately 3 kb band detected by the COX I probe in lane a (corresponding to NCN10 mitochondrial RNA) appears to be a degradation product, since it is not present in the lanes containing NCN5 mitochondrial RNA (lanes b-e), and has not been detected in previous studies using DNA from the same region of mtDNA as a probe (Burger et al., 1985). A product that is also approximately 3 kb was detected with the COX I + COB probe (lanes c-e). This is most likely an unspliced intermediate of the COB transcript, as a band of similar size has been detected by previous workers using the COB gene as a probe (Burger et al., 1985; Kubelik et al., 1990). As expected, COX III (~1.5 kb) and COB (~2.1 kb and ~1.5 kb) transcripts are also detectable in the mutant.

The finding that the COX I transcript is present at wild-type levels in the *cya-5* mutant is strong evidence against the *cya-5*$^{+}$ gene product being involved in regulation of COX I gene expression at the transcriptional level. Transcripts from the cytochrome *b* gene, which are expressed from the same promotor as the COX I gene (Kennell and Lambowitz, 1989; Burger et al., 1985), were also detected in *cya-5*, providing further evidence that *cya-5* does not have a defect in transcription. These results support the notion that the *CYA-5* protein is necessary either for translation of the COX I message, or a post-translational step, perhaps being required for stability of the polypeptide.

Conclusion

The previous finding (Nargang et al., 1978) that the *cya-5* nuclear mutant is specifically deficient in the mitochondrial encoded subunit 1 of cytochrome *c* oxidase (COX 1) was confirmed in the present study. The two *cya-5* strains examined were completely deficient in the COX 1 protein. The defect in the mutant was analyzed further by examining the levels of COX 1 mRNA in *cya-5* mitochondria as compared to a wild-type strain. Northerns of mitochondrial RNA revealed that normal levels of the mature COX 1 transcript were present in the mutant. This suggests that *cya-5* is not deficient in transcription of the COX 1 gene, or in a post-transcriptional RNA processing function, such as stabilizing the COX 1 transcript (there are no introns in the *N. crassa* COX 1 gene). This conclusion is further supported by the presence (at apparently normal levels) of the cytochrome *b* (COB) transcript in *cya-5*, which is transcribed from the same promoter as COX 1 (Kennell and Lambowitz, 1989; Burger et al., 1985). Thus, the defect in *cya-5* appears to be at the translational or post-translational level. To distinguish between these possibilities, pulse-chase labelling experiments could be performed in an attempt to detect a potentially unstable polypeptide in the mutant.

CYA-5 represents the first nuclear gene product identified in *N. crassa* that regulates the expression of a specific mitochondrially encoded gene. That the *CYA-5* protein affects COX 1 expression post-transcriptionally is not surprising, since in yeast all characterized cases of mitochondrial gene specific regulation by the nucleus occur at the post-transcriptional level (Costanzo and Fox, 1990; Fox, 1986). Indeed, gene specific regulation of transcription may not exist in mitochondria, because, among other reasons, genes are transcribed as polycistronic units (Kennell and Lambowitz, 1989).

The *cya-5*$^+$ gene was previously cloned by complementation of the mutant in transformations with a wild-type cosmid DNA library. A 6.4 kb SphI-XmaI fragment of nuclear DNA rescuing the *cya-5* mutant was subcloned into the vector pUC19, and the majority of it was sequenced. In this study, the sequencing of the cloned insert was completed, and examination of the sequence revealed a 1136 amino acid open reading frame, which most likely encodes the *CYA-5* protein. Evidence for this comes from sequence of a clone isolated from a wild-type cDNA library using the SphI-XmaI fragment as a probe. This was the only kind of cDNA that was isolated, and it was identical and colinear to most of the 1136 amino acid ORF (Fig. 7). This is also good evidence that there are no other genes encoded in the subcloned fragment used as a probe.

Additional evidence to suggest that the 1136 amino acid ORF encodes the *CYA-5* protein is provided by transformation experiments with restriction enzyme digested pJC2. Digestion with EcoRI, KpnI and SspI destroyed the ability of the plasmid to rescue *cya-5* spheroplasts, suggesting that these enzymes have sites within the *cya-5*$^+$ gene. As expected, these restriction sites lie within the 1136 amino acid ORF, the only contiguous ORF large enough to contain these sites. Transformations with SnaBI digested DNA, a restriction site that lies outside the 1136 amino acid ORF, resulted in a considerably larger number of rescued transformants, although the efficiency was reduced in comparison to controls of undigested or XmaI digested DNA (Table 5).

Further analysis of the genomic DNA sequence has provided evidence that the methionine codon at position 1289 (Fig. 8) is the *cya-5*$^+$ translation initiation site. DNA sequence immediately upstream of this codon resembles the consensus sequence established for *N. crassa* (Gurr et al., 1987; Ballance, 1986). This is also the first methionine codon in the 1136 amino acid ORF (only 37 nucleotides downstream of a stop codon), which is a characteristic typical of eukaryotic translation initiation sites, since translation in almost all eukaryotic genes begins at the first methionine codon of an open reading frame (Kozak, 1983; 1989). Moreover, sequence indicative of a mitochondrial targeting peptide follows this methionine (Gavel and von Heijne, 1990). No other region in the ORF resembles such a sequence. Since most mitochondrial targeting peptides are located in the amino terminus of proteins (Hartl et al., 1989), this adds further weight to the argument that the ATG at 1289 is the *CYA-5* translation initiation site.

If this be the case, the deduced *CYA-5* protein is 1136 amino acids long and the gene contains no introns. This is not unusual, since many *N. crassa* genes lack introns (eg. *pyr-4*, Newbury et al., 1986; *qa-1F*, Giles et al., 1985; *qa-2* and *qa-3*, Alton et al., 1982; *qa-4*, Rutledge, 1984; *cys-3*, Fu et al., 1989; *trp-1*, Schechtman and Yanofsky, 1983). The majority of other genes only contain 1 or 2 introns, some of which have long uninterrupted exons (eg. *his-3*, Legerton and Yanofsky, 1985; *qa-1S*, Huiet and Giles, 1986; *am*, Kinnaird and Fincham, 1983).

The 5' end of the *cya-5*$^+$ transcript and, therefore, the extent of its untranslated leader sequence has not been defined in this study. However, using two primers derived from sequences upstream of the putative translation initiation site, RDH5 and RDH1 (Figs. 8 and 10), the expected product was amplified from the RACE mixture containing *cya-5*$^+$ cDNA template by PCR (Fig. 12, lanes a-c). This result suggests that the *cya-5*$^+$ transcript extends at least as far upstream as the RDH5 primer, which is 738 nucleotides upstream of the putative translation start site at position 1289 (Fig. 8). This predicts an untranslated leader sequence which is unusually long compared to major transcripts of most *Neurospora* genes, which are usually less than 200 nucleotides long (Gurr et al., 1987). However, almost all *Neurospora* genes contain more than one transcription initiation site (Gurr et al., 1987). The upstream sites are often infrequently used (minor sites), and can generate leaders as long as 700 nucleotides (eg. *qa-3*). The *pho-4* gene of *N. crassa* is transcribed from 2 different major transcription initiation sites; the upstream site generates a leader that is over 1 kb long (Mann et al., 1989). It is, therefore, possible that PCR analysis detected a *cya-5* transcript generated from a secondary, upstream site of transcription initiation, which may be a major or minor site. There may be a downstream site of initiation that produces a shorter untranslated leader. In this regard, knowledge of the size of the mature *cya-5*$^+$ transcript would have been beneficial, but the message was undetectable in Northerns.

Although it has been demonstrated that the *CYA-5* protein affects the expression of COX 1 at the translational or post-translational level, nothing is known about its mechanism of action. If it acts to promote translation of COX 1, it may be functionally analagous to the yeast mitochondrial gene specific translational activators. The way in which these proteins promote translation is not well understood, but it has been established that they mediate their effects by interacting directly or indirectly with the 5' untranslated regions of mitochondrial transcripts (Fox, 1986; Costanzo and Fox, 1990). Mitochondrial mRNAs of yeast and *Neurospora* have features that suggest that they may be extensively regulated at the level of translation. These are features shared among several eukaryotic nuclear encoded mRNAs that have been shown to be similarily regulated at the level of translation (Logan and Shenk, 1984; Kirk and Kirk, 1985; Klemenz et al., 1985; McGarry and Lindquist, 1985; Hultmark et al., 1986; Katz et al., 1986; Tzamarias et al., 1986; Mueller et al., 1987; Werner et al., 1987; Rao et al., 1988). The characteristics include unusually long 5' untranslated leader sequences, and the presence of one or more short reading frames in the leader sequences (Hunt, 1985; Kozak, 1989). They also often possess regions of potential secondary structure in their leader sequences. The upstream ORFs and secondary structures have been demonstrated to function in repressing translation in several eukaryotic genes (Hunt, 1985; Tzamarias et al., 1986; Mueller et al., 1987; Werner et al., 1987). The transcript of the COX 1 gene of *N. crassa* has these features. It contains an untranslated leader sequence over 1kb long (de Vries et al., 1985; Burger et al., 1982). This leader contains a long unidentified reading frame (URF U) of unknown function. There are also regions of secondary structure in the COX 1 leader both inside and outside the unidentified reading frame. Both URF U and the secondary structures may regulate translation of COX 1 mRNA, perhaps playing a role in repression. If *CYA-5* functions as a translational activator, it is interesting to speculate that it may mediate its effects by interacting directly or indirectly with a protein encoded by URF U and/or the upstream secondary structures.

To investigate the possible mechanism of action of the *CYA-5* protein, future studies can be conducted along the same lines as investigations on yeast mitochondrial translation or post-translational factors. For example, it would be essential to show that the *CYA-5* protein is located in mitochondria, since it is conceivable that *CYA-5* never enters mitochondria, but mediates its effects indirectly by activating another nuclear gene. Sequence that is similar to a mitochondrial targeting peptide immediately downstream of the putative translation initiation site argues against this hypothesis, but definitive proof would require the use of antibodies to detect the cellular location of the protein.

If the *CYA-5* protein is a translational activator that interacts with the COX 1 leader sequence, one might expect to isolate mitochondrial revertants of *cya-5* mutations that involve rearrangements that replace the leader sequence of COX 1 with the leader of another mitochondrial gene (as observed in yeast, see introduction). If mitochondrial suppressors of this type could be successfully isolated, they would be strong evidence that *CYA-5* has a function in translation. However, it may not be possible to isolate such revertants, since *N. crassa* strains with extensive deletions in mtDNA, even when present in a heteroplasmic state with wild-type mtDNA, may not be viable.

Nuclear second site suppressors may also be informative in regards to the mecanism of *CYA-5* function. They may identify other proteins that interact, perhaps physically, with the *CYA-5* protein. For instance, *CYA-5* may associate with ribosomal proteins to activate translation. Mutations in certain genes for mitochondrial ribosomal proteins may suppress specific *cya-5* mutant alleles as demonstrated for the PET122 translational activator of yeast (Haffter et al., 1991). This may be a general mechanism of action of mitochondrial mRNA specific translational activators.

Tables

Table 1. *N. crassa* strains.

Strain	Genotype	Source
NCN5	*cya-5-34, a*	Dr. H. Bertrand
NCN10	*nic-1, al-2, A*	Dr. H. Bertrand
NCN45	*[mi-3], pan-2, A*	Dr. A. Lambowitz
NCN69	*cyt-2-1, pan-2, a*	Drygas et al., 1989
NCN80	*cya-5, nic-1, al-2*	NCN10 x NCN5

Table 2. Bacterial strains.

Strain	Genotype	Reference
JM83	r-, ara, Δ(lac-proAB), rpsL, thi, ø80, lacZΔM15, hsdR$^-$	Yanisch-Perron et al., 1985
JM103	Δ(lac-proAB), supE, thi, strA, sbcB15, endA, [F' traD36, proAB, lacI^q, ZΔM15]	Messing, 1983; Yanisch-Perron et al., 1985
XL1-Blue	endA1, hsdR17, (r_k^-, m_k^+), supE44, thi-1, lambda$^-$, recA1, gyrA96, relA1, (lac$^-$), [F' proAB, lacI^q, ZΔM15, Tn10(tet^R)]	Predigested lambda ZAP/ *Eco*RI-Instruction Manual, Stratagene, 1987

Table 3. Plasmid and cosmid vectors.

Vector	Antibiotic resistance	References
pUC19	Amp^R	Yanisch-Perron et al., 1985.
pSV50	Amp^R, Ben^R	Vollmer and Yanofsky, 1986.
pBluescript	Amp^R	lambda ZAP/EcoRI Instruction manual, Stratagene, 1987.
pBS	Amp^R	Stratagene® 1992 catalog.

Table 4. Recombinant plasmids.

Plasmid	Parental plasmid	Cloned fragment
pJC2	pUC19	~6.4 kb SphI-XmaI fragment carrying the *cya-5*$^+$ gene isolated from a *N. crassa* genomic library.
pRDC1	pBluescript	2.7 kb *cya-5*$^+$ cDNA clone with EcoRI linkers.
pRDC2	pBS	865 bp *cya-5*$^+$ cDNA clone amplified by the RACE procedure.

Table 5. Numbers of rescued transformants of NCN80 on benomyl plates.

The NCN80 mutant strain was transformed with the appropriate DNA (indicated in left column) and the number of fast growing colonies (appearing after 48 hours) were counted. These colonies were rescued transformants. The first two rows show results of negative control experiments where either pJC2 or pSV50 are left out of the transformation mixture. The third row is a positive control, as pJC2 was undigested. The remaining rows indicate the number of transformants obtained with co-transformations involving pSV50 and pJC2 which was digested with different restriction enzymes (indicated in brackets). Before the transformations were carried out, a fraction of each pJC2 digest was electrophoresed on a 0.8% agarose gel and observed to ensure that the digestion was complete, such that there was no undigested plasmid detectable. For each transformation, 0.5 µg of each plasmid was added to approximately 2.63×10^6 viable NCN80 sphaeroplasts.

Type of DNA	No. of rescued transformants
pJC2	0
pSV50	0
pJC2 + pSV50	2556
pJC2 (EcoRI) + pSV50	3
pJC2 (KpnI) + pSV50	4
pJC2 (SnaBI) + pSV50	151
pJC2 (SspI) + pSV50	0
pJC2 (XmaI) + pSV50	820

Table 6. Codon usage in *cya-5* compared with other *N. crassa* genes.

Numbers indicate the number of times each codon is used in the *trp-1* (Schechtman and Yanofsky, 1983), *am* (Kinnaird and Fincham, 1983), *tub-2* (Orbach et al., 1986) and *cya-5* (this study) genes.

aa	codon	trp-1	am	tub-2	cya-5	aa	codon	trp-1	am	tub-2	cya-5
Phe	TTT	7	1	0	12	Ser	TCT	14	3	6	8
	TTC	15	17	25	31		TCC	20	21	24	15
Leu	TTA	0	0	0	6		TCA	1	0	0	7
	TTG	10	1	2	24		TCG	8	1	0	18
Leu	CTT	21	13	6	26	Pro	CCT	11	3	2	11
	CTC	26	22	25	35		CCC	20	10	17	18
	CTA	2	1	0	6		CCA	5	0	0	12
	CTG	18	1	1	22		CCG	4	0	0	10
Ile	ATT	11	6	4	9	Thr	ACT	5	8	6	8
	ATC	33	12	9	26		ACC	22	8	17	21
	ATA	0	0	0	5		ACA	2	0	0	16
Met	ATG	15	9	20	34		ACG	5	0	0	14
Val	GTT	11	14	10	14	Ala	GCT	19	15	13	24
	GTC	36	19	23	26		GCC	37	36	16	41
	GTA	3	0	0	5		GCA	5	0	0	14
	GTG	13	0	3	17		GCG	7	1	1	33
Tyr	TAT	6	0	1	12	Cys	TGT	0	1	0	2
	TAC	10	15	13	29		TGC	10	5	7	6
Ter	TAA	1	1	1	1	Ter	TGA	0	0	0	0
	TAG	0	0	0	0	Trp	TGG	5	8	4	20
His	CAT	5	0	4	7	Arg	CGT	12	8	10	18
	CAC	13	10	6	17		CGC	12	9	11	16
Gln	CAA	7	0	3	21		CGA	0	0	0	9
	CAG	23	15	19	30		CGG	3	0	0	12
Asn	AAT	4	1	1	15	Ser	AGT	4	0	0	7
	AAC	20	22	24	33		AGC	12	3	1	14
Lys	AAA	1	0	0	9	Arg	AGA	4	0	0	15
	AAG	43	29	13	68		AGG	6	0	2	9
Asp	GAT	16	3	9	19	Gly	GGT	18	31	21	11
	GAC	24	12	12	40		GGC	34	23	16	28
Glu	GAA	3	0	1	26		GGA	7	0	0	6
	GAG	48	36	38	65		GGG	3	0	1	4

Figures

Figure 1. The electron transport chain of *Neurospora* mitochondria. The alternate oxidase pathway is present in mitochondria that have defects in components of the electron transport chain (Lambowitz and Slayman, 1971; Tzagoloff, 1982).

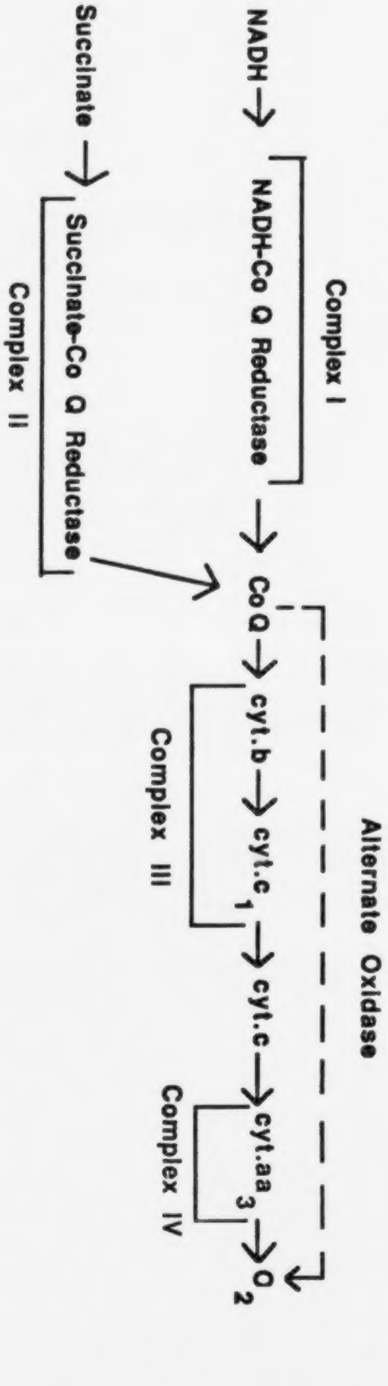
Alternate Oxidase
Complex I
NADH
NADH-Co Q Reductase
Succinate
Succinate-Co Q Reductase
Complex II
CoQ
cyt.b
cyt.c$_1$
Complex III
cyt.c
cyt.aa$_3$
Complex IV
O$_2$

Figure 2. Automatic excision of cDNA inserts from lambda ZAP vector.

The figure was modified from the lambda ZAP/EcoRI-Instruction Manual provided by Stratagene (1987). Lambda ZAP consists of lambda arms and pBluescript vector sequences. The cDNA inserts are in the cloning site of the pBluescript vector. Excision of cDNA inserts occurs after simultaneous infection of *E. coli* cells with lambda ZAP recombinant phage and M13 (or f1) helper phage. Proteins from the helper phage recognize the F1 initiation and termination sequences for DNA replication placed in the lambda ZAP vector (open square and circle, respectively) and duplicate the DNA region between the sequences. The resulting single stranded DNA molecule is circularized by gene II product of the helper phage and forms pBluescript SK(M13-) plasmid which contains the cDNA insert.

Abbreviations: A-J, lambda structural genes; att, lambda attachment site; T3/T7, RNA polymerase promoters; Amp, the ampicillin resistance gene; lacZ, the *E. coli* ß-galactosidase gene.

Figure 2 has been removed due to the unavailability of copyright permission.

Figure 3. Mitochondrial translation products of *cya-5* and wild-type strains.

Mitochondrial translation products from *cya-5* mutant strains NCN5 and NCN80, as well as wild-type strain NCN10, were labelled with L-[4,5-^{3}H]leucine and isolated as described by Bertrand and Werner (1977). The products (100 000 counts of each sample) were electrophoresed on a SDS polyacrylamide gel (see Materials and Methods), which was dried and autoradiographed. Exposure time was 15 days. Faint lower molecular weight bands are other unidentified mitochondrially encoded proteins.

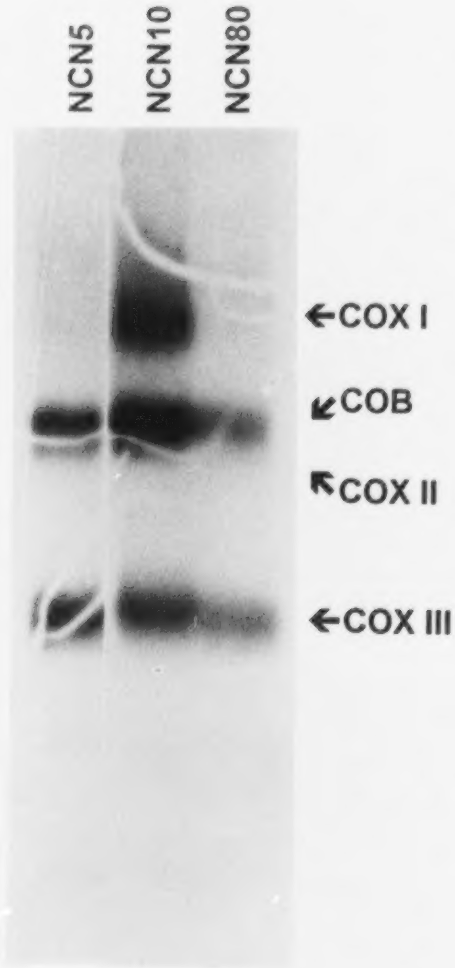
NCN5
NCN10
NCN80
COX I
COB
COX II
COX III

Figure 4. Map of cosmid vector pSV50.

The thin lines represent sequences derived from pBR322. Darkened box indicates the 1.8 kb ø80/lambda hybrid fragment containing cos sequence. The open boxes represents the 2.58 kb SalI fragment of *N. crassa* DNA containing the ß-tubulin gene (*tub-2*) that confers benomyl resistance (Ben^{R}) and the *E. coli* ampicillin-resistance gene (Amp^{R}). Arrows indicate direction of transcription of the ampicillin-resistance and ß-tubulin genes. The restriction sites indicated are: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; P, PstI; S, SalI.

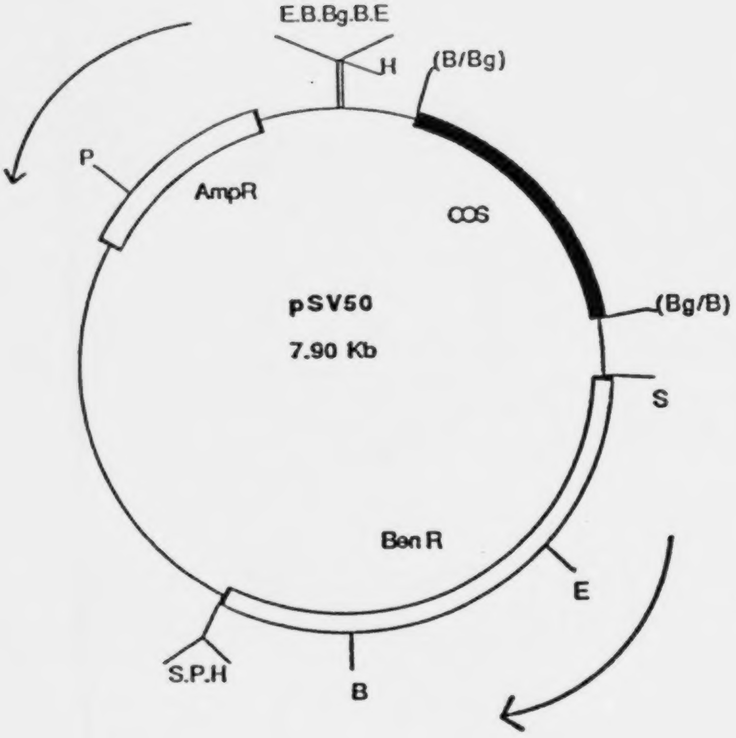
E.B.Bg.B.E
H
(B/Bg)
P
AmpR
COS
pSV50
7.90 Kb
(Bg/B)
S
Ben R
E
S.P.H
B

Figure 5. Restriction sites and ORFs in the SphI-XmaI restriction fragment that rescues the *cya-5* mutant.

All ORFs of 50 amino acids or greater in the 6 reading frames on both strands are indicated. ORFs belonging to the same reading frame are shown in the same row in the diagram. Methionine codons are represented by vertical lines on the arrows. The direction of the ORFs is indicated by the arrows, with the tail of each arrow signifying the beginning of each reading frame. Indicated restriction sites occur only once in the region. "*" indicates restriction sites that are within the *cya-5*$^+$ gene as determined by transformation experiments involving mutant rescue. Numbers indicate distance from SphI site in base pairs.

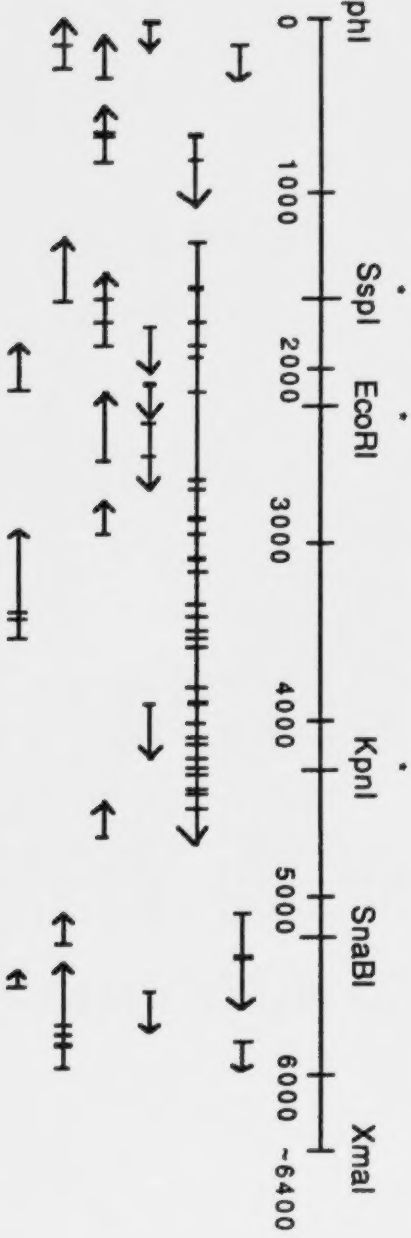
SphI
SspI
EcoRI
KpnI
SnaBI
XmaI
0
1000
2000
3000
4000
5000
6000
~6400

Figure 6. Co-transformation of cosmid vector pSV50 and restriction enzyme digested plasmid pJC2 into NCN80 spheroplasts.

Plasmid pJC2 (carrying the *cya-5*$^+$ gene) was digested with various restriction enzymes (eg. enzyme "R"), and co-transformed with undigested pSV50 (carrying benomyl resistance) into NCN80 (*cya-5*) spheroplasts. Cells were plated onto benomyl containing media and incubated at 30°C. Rescue of the mutant was observed as fast growing, benomyl resistant colonies after 48 hours, in a background of non-rescued, benomyl resistant colonies that appeared after 72 hours incubation.

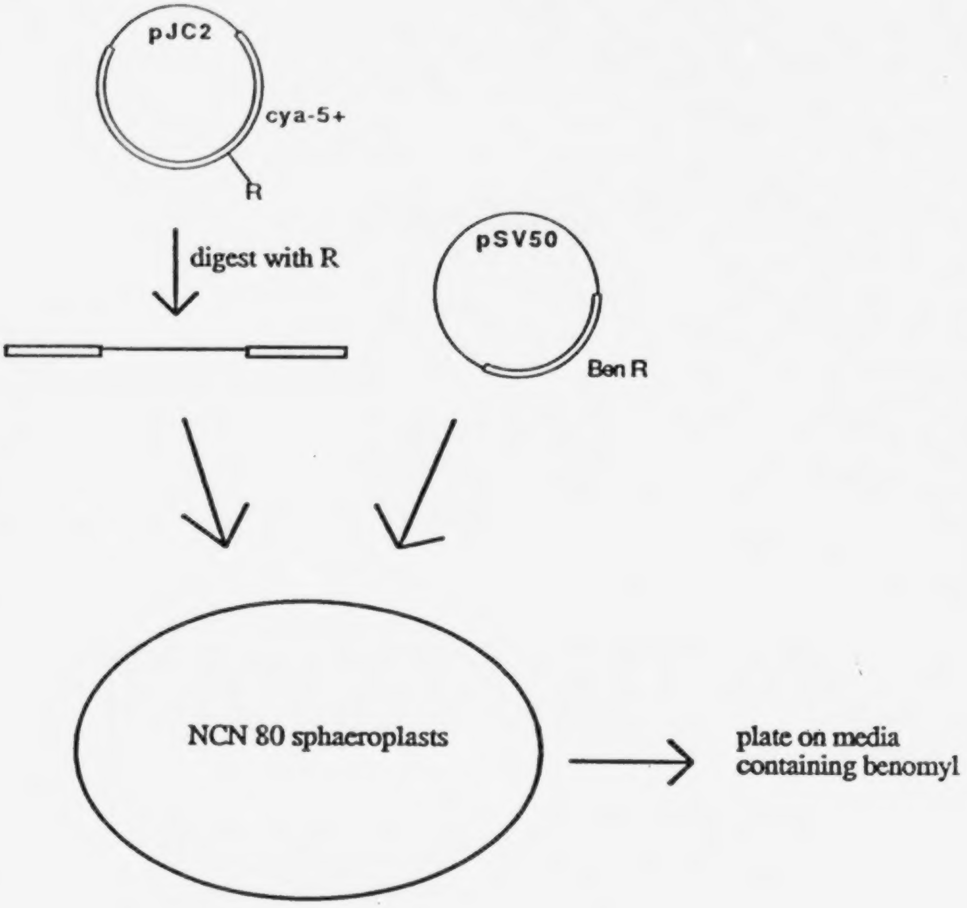
pJC2
cya-5+
R
digest with R
pSV50
Ben R
NCN 80 sphaeroplasts
plate on media
containing benomyl

Figure 7. Location of the *cya-5*$^+$ ORF and cDNAs.

The first methionine codon in the 1136 amino acid ORF (at position 1289) is shown. This methionine codon exhibits the strongest similarity to a *N. crassa* translation start site, and is immediately followed by a potential mitochondrial targeting peptide sequence. All numbers indicate distance in bp from SphI site.

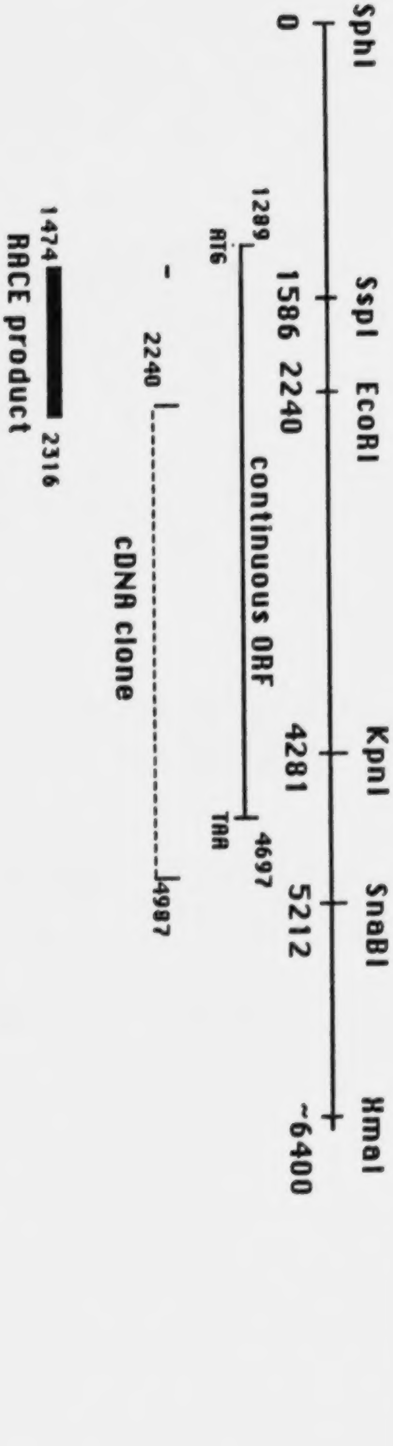
SphI
0
SspI
1586
EcoRI
2240
KpnI
4281
SnaBI
5212
XmaI
~6400
1289
ATG
continuous ORF
4697
TAA
2240
cDNA clone
4987
1474
2316
RACE product

Figure 8. DNA sequence of the SphI-SnaBI restriction fragment that contains the *cya-5*$^+$ gene, and the predicted amino acid sequence of the *CYA-5* protein.

The predicted 1136 amino acid sequence of the *CYA-5* protein is indicated below the corresponding nucleotide sequence. ↓ and ⇩ : The 5' and 3' ends, respectively, of the cDNA clone obtained from the lambda ZAP cDNA library. * : The 5' end of the cDNA obtained by the RACE procedure. Bases underlined once indicate the annealing sites of the 5' amp and 5' RT primers used in the RACE procedure, as well as control primers (RDH1, RDH5 and RDH22) used in PCR reactions. Bases underlined twice indicate the annealing site of the adaptor primer used in the RACE procedure. Bases underlined by dashed lines indicate possible poly A signal sites. X : Nucleotides not identical to the adaptor primer. → and ← : Direction of extension of the RACE primers. Amino acids highlighted in upper case letters belong to a predicted amino terminal mitochondrial targeting peptide.

```
GCATGCGAAGCCTTCATGGACATGGCATGAGCCCATGTGCGCTCGGCCGTGAGAAGCGCAA 61
 SphI
GGTGGAGGTATCTGAGAGTAGCACACGAGACGAGGTTAGCAGGACGGCCGGCAAGCATTG  121
CGCCACCACGATAAGCACAAGAGAGAGTCGTACTCATGGTTGTCCGAGATTTGCTCAGCG  181
GTAATCTGGGCCTTGGGGTGGAATTTGCCTCGGCTCTTGGTCGCAATGTTGAGCTTCTTG  241
CGGGAGTTAAGAAGCTTCCTGGAGAGTTGGGCACGATACGTCGCATAGTCACCGTAGAGG  301
AGGGCGTTCTCCCGGTGAGATACCACGAACTTTGTGATATCCATGGTGTTTGTTGAGGTT  361
GAATATGTGTAGTGTGTGTGTAAGTAAGGTGATGCGCTCCGGTCGCAACTAGAGGGTC  421
CTACAGAAAAGTGTTAAGCTATGAAGCACCCGTTGCGCAACTGGGCGTAGGGCAGGTAGC  481
TTCGATCAAAGCCGTGGTGGTATAGGTGTAAAGAGCAGTTGGAGACGGCCTCCTTGTACG  541
          RDH 5 →
TCTGGCGGCTTGTTGAGTCTCGACGTCGAGGTTGAACCTGGAAAGTGTGGGGCGTGGGCA  601
GCTCTCTCTAATGGAGCACCGTATGTTACTTGCCCGTGTTGTCCCCAATTGCCCCGCATG  661
AGATGGATGGAAGCCATAGTGACCTGGCCAGCATGGAACAGCTGGCAGCAGTCGGATTTC  721
AGTGATTGGCACCCCCGACGAGATCTGAGATACCTACCTATAGTGTACCTAAGGGATCCA  781
CCGGACCAGGAGAGCTCACACGGCCGCCTCTGCATGGACGTTGCTGGGACAGAAGCTTTT  841
TGGACGGCGACGCGGGCGGGCTGGGCGGGCGGCCATGTCGTAGGTGAGGAGCCCCACTTT  901
CGCGCTGTGAAATGGTCGAATCGCGCTCAAAAATTTTGGTTGGTGCTTGCGACAGCCAAA  961
GTCTTGTCTTTCAAGGTATTTTTTGCTGTTAGTCCCGCGAATTGTGACCGAGCAACCCGA  1021
CAGCGTTGTTTTCTCTTACCCCGATTTGGAAGCAATCCTAGCTCTCATACGACGTAGCTT  1081
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```
TGCGAAGCTATTCCCTCCCTCCCAAACGCCCACAGACTCTATTCCAGAACGCCCATCCCG  1141

GTTCGAACATTCCCTGATCAAGGAACACAAGCCGAGACGAGCGAGGGACAAACAAAACGA  1201
                                              ← RDH 1
GAGCGGAACATGCCCGAGAGAGACGACCAACACCTACGACTCACACATTAACTACGCCAG  1261

CTGGCTGGGCCTCCGCCTTCCAACGCGATGCTGGAGAGGACGGCGGCGAGCCTCGAAACA  1321
                           METLeuGluArgThrALAALASERLEUGLUTHR

TGCAGCCTCCAGAGAGTTCTCCCCGTCGCCAGAACCTCGCTGAAGAGCCAGCGACAACTA  1381
CYSSERLEUGLNARGVALLEUPROVALALAARGTHRSERLEULYSSERGLNARGGLNLeu

CACACAGGCTTTTGGCAACATGGCGCCTCCGACCTTGAGCTCCTCGATGCCTACCAGACC  1441
HisThrGlyPheTrpGlnHisGlyAlaSerAspLeuGluLeuLeuAspAlaTyrGlnThr
         *      XXX     X   X →
CTCCTGCGAGACTCGACACCGACACCGACGCAGAACCTTGGTAGCTCGCAGACGACCAAG  1501
LeuLeuArgAspSerThrProThrProThrGlnAsnLeuGlySerSerGlnThrThrLys

ACCGAAAGCAGAATAGAGCCCATGAAGGCGTCCATGTTCTTGCTCGACTTCCTATACCCA  1561
ThrGluSerArgIleGluProMETLysAlaSerMETPheLeuLeuAspPheLeuTyrPro

AGTGGAACCGCCGCTCTTCTTCGGAATATTCTCCCCATTCGACTACCATCCCTCGAATCG  1621
SerGlyThrAlaAlaLeuLeuArgAsnIleLeuProIleArgLeuProSerLeuGluSer

GCGCACAAGACTCGTAGAAATGCGACGCGCTTATACAGTTCAGCGATAAATAACAACTCC  1681
AlaHisLysThrArgArgAsnAlaThrArgLeuTyrSerSerAlaIleAsnAsnAsnSer

GGCGCTGCGCAAGAACCGCAAGAGAAACCGCAAGCGGACGACTCGTTCATGAACGCCGAC  1741
GlyAlaAlaGlnGluProGlnGluLysProGlnAlaAspAspSerPheMETAsnAlaAsp
                                    RDH 22 →
CTTTTCGAACAGTACTGGAATGAGCAGGAGAGTGGCGTCGTCCAAGACAGGAGACCACAG  1801
LeuPheGluGlnTyrTrpAsnGluGlnGluSerGlyValValGlnAspArgArgProGln

AAGGTTTCCAAAAAATCTGGCCCTCGGGTACTGCGGGAGCTTCTCGAGCCGTCAGAGTTC  1861
LysValSerLysLysSerGlyProArgValLeuArgGluLeuLeuGluProSerGluPhe
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GGTCCCATGTACGAGCAGATATGGACGGCTTACAACAAGCTCGAGCCCAAGGAGCAGGAG  1921
GlyProMETTyrGluGlnIleTrpThrAlaTyrAsnLysLeuGluProLysGluGlnGlu

GATTTCAAGGGCGATGTCATGGTCAACTTTGCGCAGTCTGGTCGGTCGGTTGATGCTTGG  1981
AspPheLysGlyAspValMETValAsnPheAlaGlnSerGlyArgSerValAspAlaTrp

AGGATCCGAGAGCTGTTCGCCACCGTGCCTGTTGAGCGGTGGACAGAATCCATAATCCAA  2041
ArgIleArgGluLeuPheAlaThrValProValGluArgTrpThrGluSerIleIleGln

GCCGCTATCAAGGCCGAACTTGCCTTGGACGACGTGGACAATGCAAAGGTGCTGTTTTCC  2101
AlaAlaIleLysAlaGluLeuAlaLeuAspAspValAspAsnAlaLysValLeuPheSer

GATGCCCTCAAGAAGAGGGGTCTTCATCAAGGACTTGACGATATCATGGCACACGCGTTT  2161
AspAlaLeuLysLysArgGlyLeuHisGlnGlyLeuAspAspIleMETAlaHisAlaPhe

GACACCTCTTCGTGGCAGCTGGCCTTGGACGTTTGGAAGTTATTTAGCAACTTCAAGGGC  2221
AspThrSerSerTrpGlnLeuAlaLeuAspValTrpLysLeuPheSerAsnPheLysGly
                  ↓
TCTGAAAAGCTTACTGTCGAATTCAAGACATTGGCTTCCGTGCCTGGCTTCCGGGATAAG  2281
SerGluLysLeuThrValGluPheLysThrLeuAlaSerValProGlyPheArgAspLys
                 ←5'amp primer
CTAGCCGAGTTTGTCGAATATGTTGCGCCGAAGGTGCCAGCGACGAAGGCCAAAAAGTCC  2341
LeuAlaGluPheValGluTyrValAlaProLysValProAlaThrLysAlaLysLysSer

ACCAAGACCAAGTCGGTCAAGACAAAGTCTCCGGCCGATACAGAGTCTGCGCCTAGCTCA  2401
ThrLysThrLysSerValLysThrLysSerProAlaAspThrGluSerAlaProSerSer
          ←5'RT primer
GCACTTGCAGTCGATACGGAGATCGCAGATTTCGTCAGAGTCCTTGCCAACAAGTCATTA  2461
AlaLeuAlaValAspThrGluIleAlaAspPheValArgValLeuAlaAsnLysSerLeu

CGCTTATTCTCACCCACGGACGCAGTCGTTCTTCTTCGCCATGCTGCGGATCCATCGGCA  2521
ArgLeuPheSerProThrAspAlaValValLeuLeuArgHisAlaAlaAspProSerAla

TACGAGGCTTTCATCAAGCTGGCTATCGAACAGGGCAAGACCAGAGCTGCGGCGGAGGCC  2581
TyrGluAlaPheIleLysLeuAlaIleGluGlnGlyLysThrArgAlaAlaAlaGluAla
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TACAAGAAGTATCGTCAACTACCCAAGGCCGAGGTCCGTATTTGGGTGTTGCGCGCCATG  2641
TyrLysLysTyrArgGlnLeuProLysAlaGluValArgIleTrpValLeuArgAlaMET

CTCGACATCTTCTATCCTACCGACGCTGGCGGCATGGAGCTTGTCCTGAAAGATTGGTAC  2701
LeuAspIlePheTyrProThrAspAlaGlyGlyMETGluLeuValLeuLysAspTrpTyr

AGCCGCTACGACGCCCTTGAGAAGCGAGTTTATCAAAAGTTCCTGGCATTTTATGCTGGC  2761
SerArgTyrAspAlaLeuGluLysArgValTyrGlnLysPheLeuAlaPheTyrAlaGly

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GAAGCCGCGCGAGATATGTCACTCATCTCCAGTCTGATGCACGTGCACGCGCGCAACGGC  2881
GluAlaAlaArgAspMETSerLeuIleSerSerLeuMETHisValHisAlaArgAsnGly

AACCCTGAAGCGGCTCGCAAGGTGCTGGAAAACTGTTTAGAGCAGACGGGCATGGGCAAA  2941
AsnProGluAlaAlaArgLysValLeuGluAsnCysLeuGluGlnThrGlyMETGlyLys

CCAAAGACTGTTCACTGGAATATCTTGCTCAATGCTTACGCAAAAGCATCGGATTACAGT  3001
ProLysThrValHisTrpAsnIleLeuLeuAsnAlaTyrAlaLysAlaSerAspTyrSer

GGCTGTCTCGAGGCCTTTGAGCGTTTGGACGAGGTTGGAAAGCCGGACTCCTATTCCTAT  3061
GlyCysLeuGluAlaPheGluArgLeuAspGluValGlyLysProAspSerTyrSerTyr

GGAACTTTGATGGGTATGGCTGCTGTGCGTGGTGACCTCTCGTTTGCTTTGGTGCTCTTC  3121
GlyThrLeuMETGlyMETAlaAlaValArgGlyAspLeuSerPheAlaLeuValLeuPhe

AACGCCGCGAAGGCTGACGGCATCAGGCCTGATGTGGGAATGGTGGACAGCTTGATTGAA  3181
AsnAlaAlaLysAlaAspGlyIleArgProAspValGlyMETValAspSerLeuIleGlu

GCTTACTGCCAGAACGACCGATTCCGTGAGGCCGAGGTCATTGTGAAGCAGACTGTGAAG  3241
AlaTyrCysGlnAsnAspArgPheArgGluAlaGluValIleValLysGlnThrValLys

AAGGGCACACTTCCCGGCAAGTATACGGTTCTCTTTAACACCCTTCTCAATCACCACGCC  3301
LysGlyThrLeuProGlyLysTyrThrValLeuPheAsnThrLeuLeuAsnHisHisAla
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AGGAGGCGAGACCTCAAGGGGTTGTATAACCTCGTGGAATACATGCACGAGAACAAGATT  3361
ArgArgArgAspLeuLysGlyLeuTyrAsnLeuValGluTyrMETHisGluAsnLysIle

CCCAGCGACAATGAGACCTATAGCCATCTCTTGTACGGCTTGATGTACTGCAGACAGTCG  3421
ProSerAspAsnGluThrTyrSerHisLeuLeuTyrGlyLeuMETTyrCysArgGlnSer

CATCACGCGCTGAACCTTTTGCGGGCTTCCGAGAAGGACAATCTTTTCAAGCCCGCACCT  3481
HisHisAlaLeuAsnLeuLeuArgAlaSerGluLysAspAsnLeuPheLysProAlaPro

GAACACTACGTTCTCCTTATGGCCTCCTTCATTCACAGTCGTGAACCACATATGGCCCTT  3541
GluHisTyrValLeuLeuMETAlaSerPheIleHisSerArgGluProHisMETAlaLeu

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LysLeuAsnGluIleLeuSerLysArgGlnGluProGluMETAlaGluArgThrThrArg

GTCATCGACGCCTTGGGCAGATGGCAGCAGCTCCCCGGCCACCAAAGGTGGCAAAAGGAT  3661
ValIleAspAlaLeuGlyArgTrpGlnGlnLeuProGlyHisGlnArgTrpGlnLysAsp

CAAAAGTACTACATTACAAAGGCCATCAACCTGTTCAAGCAGACCCTGCAGAAGACTGGA  3721
GlnLysTyrTyrIleThrLysAlaIleAsnLeuPheLysGlnThrLeuGlnLysThrGly

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LysGlyGlnGlnGluAlaProArgProValValAsnGlnPheAlaHisValLeuPheIle

TTGACGCAGATCCGGGATTTCGGCACCATCGAAGAAATCATGGATCTATACACCACCCAA  3841
LeuThrGlnIleArgAspPheGlyThrIleGluGluIleMETAspLeuTyrThrThrGln

TTCCCATCTGCAAAGGAGCCCAGTAACTTGCCCATCCGCCTGCTCAACAATATGATGCTC  3901
PheProSerAlaLysGluProSerAsnLeuProIleArgLeuLeuAsnAsnMETMETLeu

GCCGACTATCATGAGCGCAAGTTCGACCGAGTCAGAGAGATCTGGAATCACGTTCTCAGA  3961
AlaAspTyrHisGluArgLysPheAspArgValArgGluIleTrpAsnHisValLeuArg

AAGACGACATTACAAGCCTTCGGTGAAGTCGCCGAGGAGCCACGGGAGATGGGTGCTGAG  4021
LysThrThrLeuGlnAlaPheGlyGluValAlaGluGluProArgGluMETGlyAlaGlu
```

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GGTGCGGAAACACCAGCTCAACAACTTGTCGGCCCTTACAAGTGGATCCTGTGCGACCCC  4081
GlyAlaGluThrProAlaGlnGlnLeuValGlyProTyrLysTrpIleLeuCysAspPro

ATCAAGACGATGCAGCGCGTCTTTTACGACGAGAACGACGCCGACGGCTTATGGCATTC  4141
IleLysThrMETGlnArgValPheTyrAspGluAsnAspAlaAspGlyLeuMETAlaPhe

GTCAAGGACGTCCGCAGCCGTGGCTTCGAGCTCGACAGCAAGAATTGGAACTACTACGTC  4201
ValLysAspValArgSerArgGlyPheGluLeuAspSerLysAsnTrpAsnTyrTyrVal

CAATCGCTTGCGCGCATGAACCGTTACCGCGAGGCCTTCGGCGAGTGCGAAGCGCGTCTC  4261
GlnSerLeuAlaArgMETAsnArgTyrArgGluAlaPheGlyGluCysGluAlaArgLeu

ATGCCCAACTGGACAGGCTGGTACCGGGTTCGCGCGCGGTCTGCCATGAAGAACCAGCTG  4321
METProAsnTrpThrGlyTrpTyrArgValArgAlaArgSerAlaMETLysAsnGlnLeu

CCGCTCGAGCTCCGTCGTCTGGGCTCGTTCCCTCAGCGCCCAAGACCCATCTCGCACACC  4381
ProLeuGluLeuArgArgLeuGlySerPheProGlnArgProArgProIleSerHisThr

TTGATCATCATGGCCAAGAAGTACATGGACTTGGAGAACTTGGCCAACTGGCAGAAGGAG  4441
LeuIleIleMETAlaLysLysTyrMETAspLeuGluAsnLeuAlaAsnTrpGlnLysGlu

GCAGCCAATCAGCTGGAGTGGGTAAATGCGGAGTGCCCGCTTACCATCAAGGCCGTCAAG  4501
AlaAlaAsnGlnLeuGluTrpValAsnAlaGluCysProLeuThrIleLysAlaValLys

ACCATGATCAGAGCGGACTCGGACTTGGAGAAGAGCGTCTTCAACGAGACTGAGGACGTG  4561
ThrMETIleArgAlaAspSerAspLeuGluLysSerValPheAsnGluThrGluAspVal

CTGGCGACAGAAGAGGGGTACGCTGAGAGATATGAAGACGAGGAGAACTGGTCAGAGATG  4621
LeuAlaThrGluGluGlyTyrAlaGluArgTyrGluAspGluGluAsnTrpSerGluMET

GCGGAGGGGGAGTTGAGGGAGACGGCGAAGAGAGAGCAGGCGTTTGCGAACCAGCTCATG  4681
AlaGluGlyGluLeuArgGluThrAlaLysArgGluGlnAlaPheAlaAsnGlnLeuMET

TCGAAACGTATTGTATAAACAGACACTTACCTTGTATAAAGATCTTTGAAGAAGTTGAGC  4741
SerLysArgIleValSTOP                -----
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                                          ----
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AAAATGGGATGGGCAGGAAAAGTGCATGGCCGGCTAGAAAAAGCCTTTAGTAGGGCACGG  4921

AGTCAGACGTGTTATTGAGGTAGAGGCACTGGGTGAAGTGCTTGGAAGGTCATCTTCTAA  4981
     ⇩
CTGGACAAGATATAACATGGCGAACAAGTAAAGCAAAGTGAACATGGGCAATATGGTACA  5041

TGGTTATTTCATGCTACCATGCTAATTAGGGGTTCGTTTTTCCTATCTATGACATGATAT  5101

GCTTTTCCCTCTTCGTTTCTTGTCTAGTTCCACCATCTGCCATCTCCTCCTCATGTCTAT  5161

CAGAAGTACGAAAAGCAAGACTGAAAACGAAACGAGACCGTTCACAACAGTACGTA  5217
                                                  SnaBI
```

Figure 9. Outline of the RACE procedure for cDNA synthesis of the 5' end of a specific mRNA.

Figure adapted from Frohman *et al.* (1988). The initial step in the procedure involves reverse transcription of poly A RNA using a gene-specific primer (5' RT). The first strand product is then separated from excess 5' RT primer and a poly(A) tail is added using dATP and terminal deoxynucleotidyl transferase. Second strand synthesis is carried out with the $(dT)_{17}$-adaptor primer. The products are then amplified with the adaptor primer and 5' RT primer through 30 cycles of PCR. A fraction of the product is re-amplified with the adaptor primer and a nested gene specific primer, 5' amp, which consists of a sequence upstream of the 5' RT primer. $\boxed{\text{5' RT}}$ The 5' RT primer. $\boxed{\text{5' amp}}$ The 5' amp primer. $\boxed{\text{****}}$ The adaptor primer. $\boxed{\text{****TTTTTT}}$ The $(dT)_{17}$-adaptor primer. 5' RT primer: ATCTGCGATCTCGTATCGACTG. 5' amp primer: ACCTTCGGCGCAACATATTCGA. Adaptor primer: GACTCGAGTCGACATCGATGCA. $(dT)_{17}$-adaptor primer: GACTCGAGTC GACATCGATGCATTTTTTTTTTTTTTTTT.

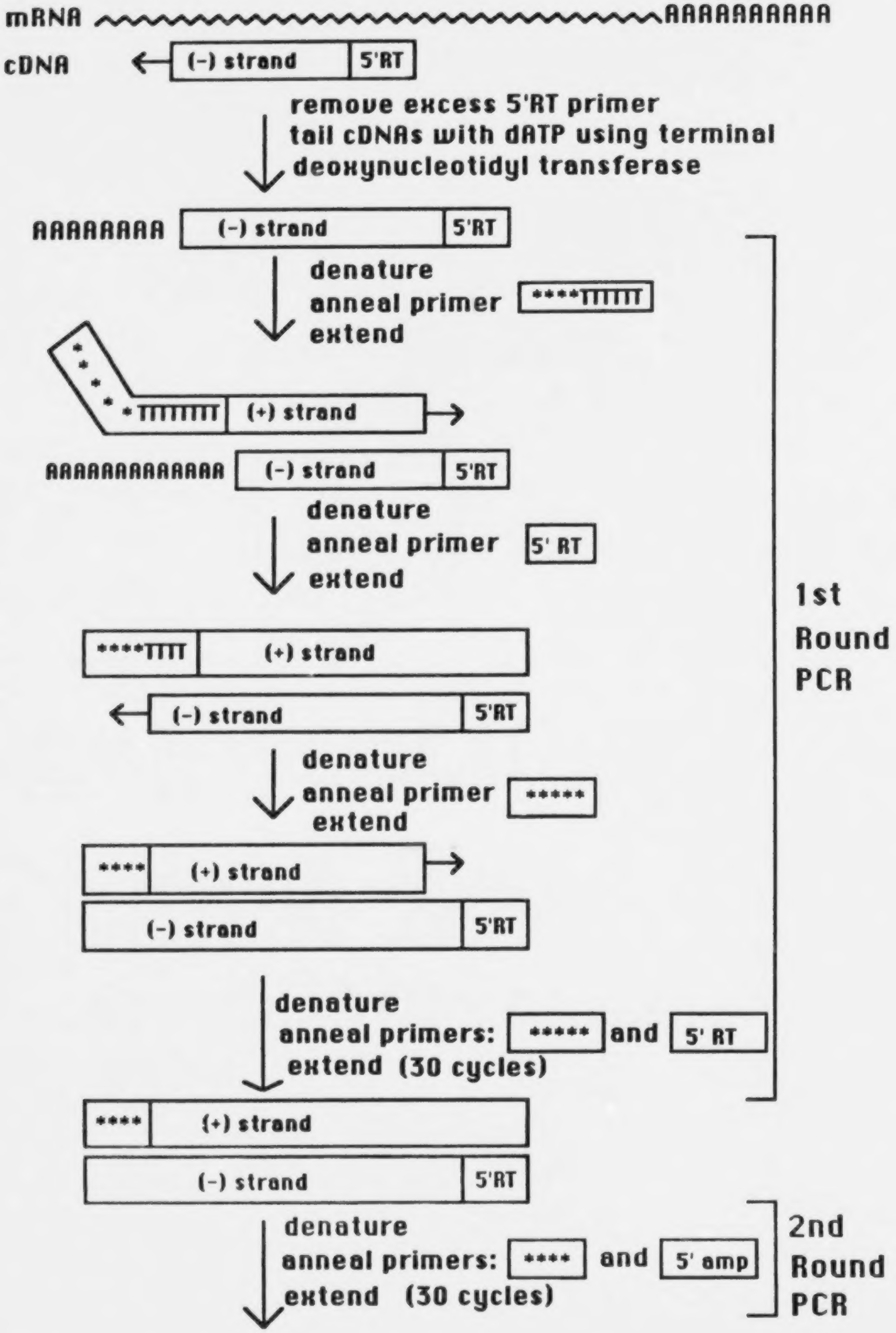

mRNA
AAAAAAAAAA
cDNA
(-) strand
5'RT
remove excess 5'RT primer
tail cDNAs with dATP using terminal
deoxynucleotidyl transferase
AAAAAAAA
(-) strand
5'RT
denature
anneal primer
****TTTTTT
extend
*
*
*
*
*TTTTTTTT
(+) strand
AAAAAAAAAAAAA
(-) strand
5'RT
denature
anneal primer
5' RT
extend
****TTTT
(+) strand
(-) strand
5'RT
denature
anneal primer
*****
extend
****
(+) strand
(-) strand
5'RT
denature
anneal primers:
*****
and
5' RT
extend (30 cycles)
1st
Round
PCR
****
(+) strand
(-) strand
5'RT
denature
anneal primers:
****
and
5' amp
extend (30 cycles)
2nd
Round
PCR

Figure 10. Location of primers used in PCR reactions.

Upward arrows indicate location of primers in the genomic fragment containing the *cya-5*$^+$ gene. Horizontal arrows indicate direction of extension of primers. Downward arrow labelled "Met." indicates the location of the methionine codon (position 1289) that is believed to be the *CYA-5* translation initiation site. Downward arrow in bold indicates the 5' end of the incomplete *cya-5*$^+$ cDNA clone obtained from a lambda ZAP library. Numbers indicate distance from the SphI site in base pairs. Only a portion of the genomic fragment (cloned in pJC2) is depicted. It does not contain the ends of the *cya-5*$^+$ cDNA and coding region.

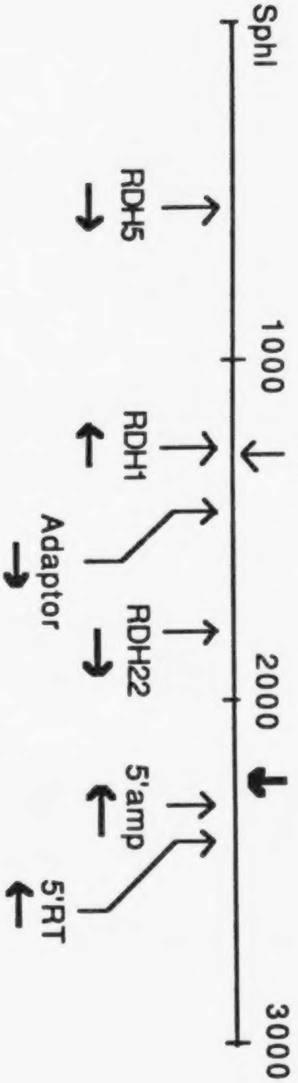

SphI
RDH5
1000
Met.
RDH1
Adaptor
RDH22
2000
5'amp
5'RT
3000

Figure 11. *Cya-5*$^+$ cDNA products amplified by the RACE procedure.

A. Lanes a and b: An ethidium bromide stained gel of products of the first round of PCR (Fig. 9). Lanes a and b differ only in the concentration of Mg^{2+} (6.8mM and 6.0 mM, respectively) used in the reactions. Other reaction conditions are described in detail in Materials and Methods. The amplified 980 bp product can be detected (although it is very faint) in lane a. Lanes c and d: Reactions in lanes a and b, respectively, amplified through a second round of PCR with a nested primer (Fig. 9). With the exception of annealing temperature (see Materials and Methods), all reaction conditions were identical to those of the first round of PCR. The band detected is 865 bp long. Lanes e and f: Southern blot of DNA in lanes c and d, respectively, probed with 2240 bp SphI-EcoRI fragment of pJC2 (Fig. 7). The probe hybridized to the 865 bp band detected in lanes c and d. m, HindIII digested lambda DNA used as a DNA size marker. Sizes are indicated in base pairs.

B. An ethidium bromide stained gel of RACE products from 2 rounds of PCR amplification. The reactions differ in the annealing temperature used in the first round of amplification. Lanes a-d: First round annealing temperatures of 45°C, 50°C, 55°C and 62°C, respectively, were used. The annealing temperature for the second round of amplification was 62°C. All other reaction conditions were identical to those described in Materials and Methods. All reactions amplified the truncated 865 bp product. m, HindIII digested lambda DNA used as a size marker. The sizes are indicated in base pairs.

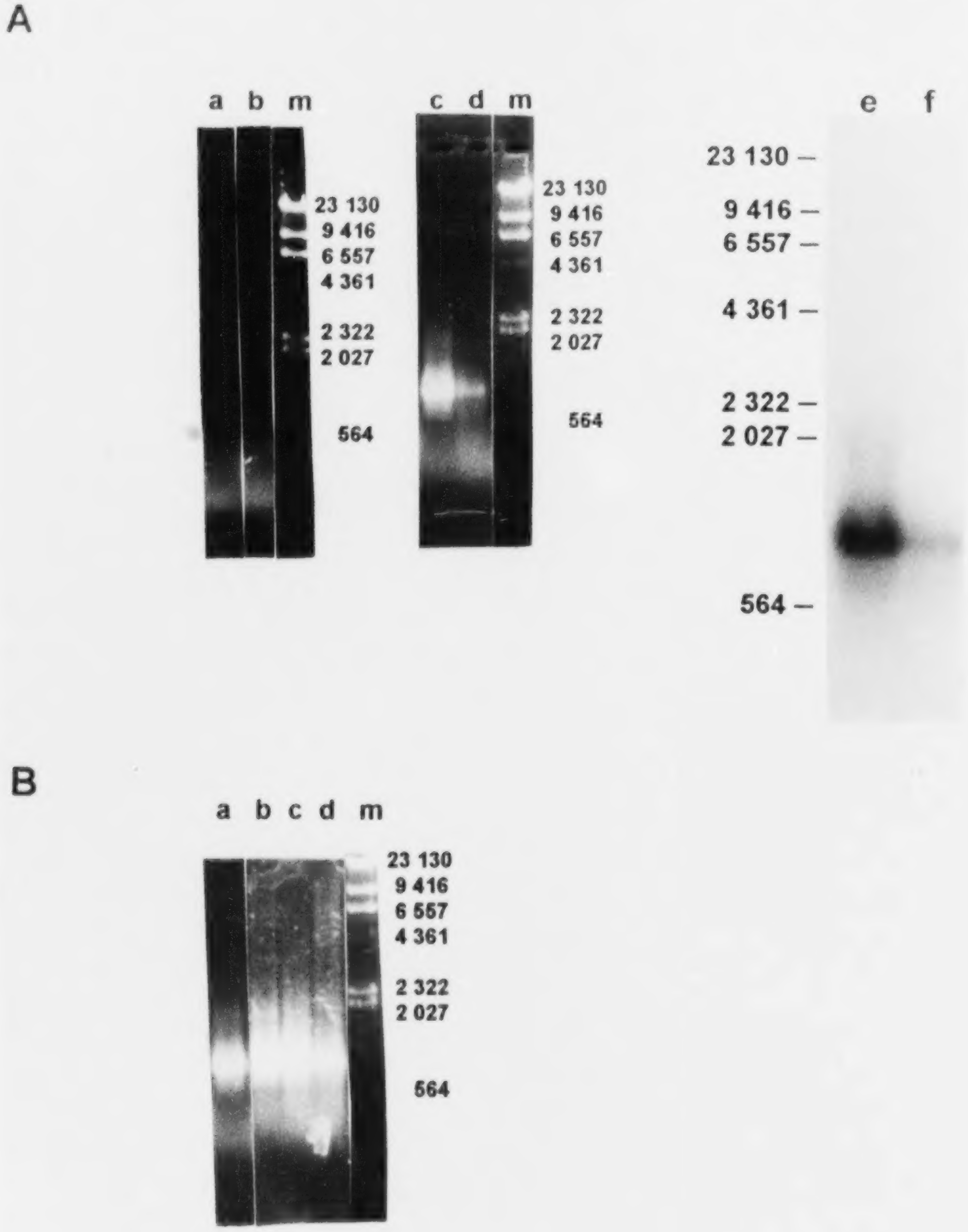
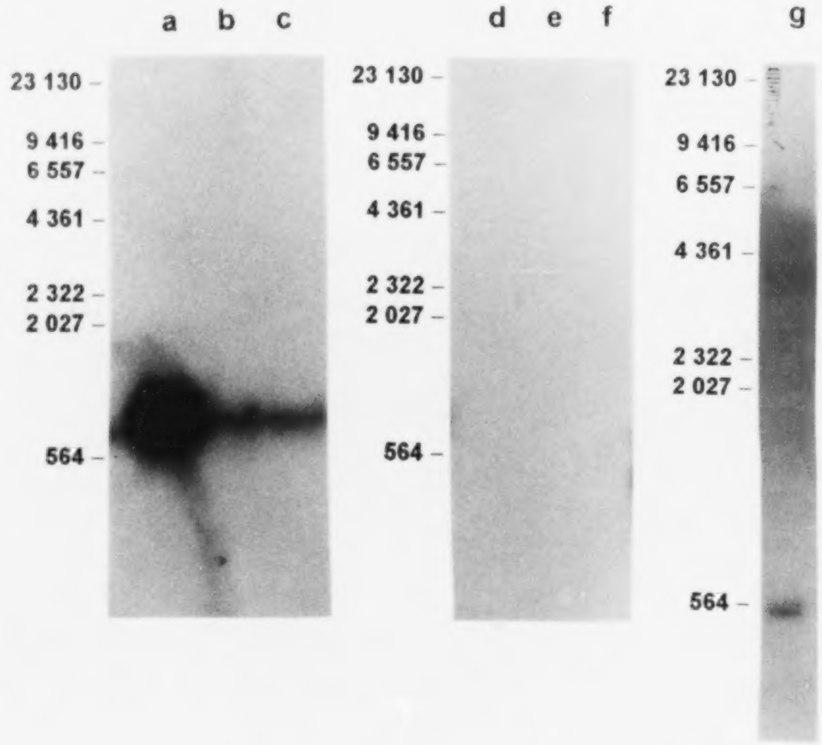

A
a
b
m
23 130
9 416
6 557
4 361
2 322
2 027
564
c
d
m
23 130
9 416
6 557
4 361
2 322
2 027
564
e
f
23 130 –
9 416 –
6 557 –
4 361 –
2 322 –
2 027 –
564 –
B
a
b
c
d
m
23 130
9 416
6 557
4 361
2 322
2 027
564

Figure 12. Evidence that the truncated RACE product is *cya-5*$^+$ cDNA and not amplified genomic DNA that may have contaminated the poly A RNA preparation.

Various primers were used in PCR reactions of the RACE mixture (consisting of wild-type poly A RNA and first strand *cya-5*$^+$ cDNA). The reaction products were electrophoresed on 0.8% agarose gels and Southern blotted. The blot consisting of reactions performed with primers THA1 and 2 (lanes d-f) were probed with an EcoRI fragment containing the *mom-19* gene (Schneider et al., 1991). The other blots were probed with the SphI-EcoRI fragment of pJC2 (Fig. 7). Lanes a-c: primers RDH1 and RDH5 (Figs. 8 and 10) used in PCR reactions at varying Mg^{2+} concentrations of 1.5mM, 2.0mM and 2.5mM, respectively. The probe detected the expected 710 bp product. Lanes d-f: as in lanes a-c, respectively, except primers THA1 and THA2 were used. Lane g: primers RDH22 and 5'amp (Figs. 8 and 10) in PCR reaction with a Mg^{2+} concentration of 2.0 mM. The probe detected the expected 542 bp product. All other reaction conditions are identical to those described in Materials and Methods. THA1 primer: TACGGGTACGATAATTACGG. THA2 primer: GCCTCCCCTTTTTCTTCTAG.



a
b
c
d
e
f
g
23 130
9 416
6 557
4 361
2 322
2 027
564
23 130
9 416
6 557
4 361
2 322
2 027
564
23 130
9 416
6 557
4 361
2 322
2 027
564

Figure 13. Similarity of sequence upstream of putative *cya-5*$^+$ translation initiation site to 8 nucleotide conserved region preceeding ATG start site codon of several *N. crassa* genes.

Sequences obtained from: a) this study; b) Schechtman and Yanofsky (1983); c) Woudt et al. (1983); d) Kinnaird and Fincham (1983); e) Arends and Sebald (1984); f) Legerton and Yanofsky (1985); g) Stuart et al. (1987), Bottorff, (1990); h) Kuiper et al. (1988); i) Paluh et al. (1988); j) Roberts et al. (1988); k) Fu et al. (1989); l) Sachs et al. (1989); m) Drygas et al. (1989); n) Newbury et al. (1986); o) Huiet and Giles (1986); p) Orbach et al. (1986); q) Alton et al., 1982; r) Rutledge, 1984; s) Mann et al., 1989; t) Fu and Marzluf, 1990.

Gene	Conserved sequence
cya-5 [a]	CCAACGCG ATG
trp-1 [b]	CAATCACA ATG
histone H3 [c]	CCATCACA ATG
histone H4 [c]	ATATCAAA ATG
am [d]	CCTTCAAA ATG
ADP/ATP carrier [e]	ATATCACA ATG
his-3 [f]	AAAACACC ATG
cyc-1 [g]	CAGTCAAA ATG
cyt-21 [h]	GGTCCAAC ATG
cpc-1 [i]	CAGTCAAC ATG
con-10 [j]	TCGTCAAC ATG
cys-3 [k]	ATGGCACA ATG
cya-4 [l]	CCGCCACC ATG
cyt-2 [m]	CAGTCGCA ATG
pyr-4 [n]	CAGCCAAC ATG
qa-1S [o]	CCGCCATC ATG
tub-2 [p]	CGGTCAAG ATG
qa-2 [q]	CAAACACA ATG
qa-4 [r]	CTTTCGCC ATG
pho-4 [s]	CGTTCAAG ATG
nit-2 [t]	GTGCGACA ATG

***N. Crassa* consensus** **CX(A/G)TC(A/G)(C/A)(C/A) ATG**

Figure 14. Northern analysis of the *cya-5* transcript.

Lanes a: 4 µg of NCN10 poly A RNA. Lanes b: 8 µg of NCN45 (*lmi-3*) mutant) poly A RNA. Lanes c: 7.5 µg of NCN69 (*cyt-2-1* mutant) poly A RNA. Lanes d: 18 µg of NCN5 total RNA. Lanes e: 18 µg of NCN10 total RNA. RNA was probed with each of 3 probes (indicated above lanes). The *cya-5* probe consisted of the 2041 bp EcoRI-KpnI fragment of pJC2 (Fig. 7). The *cyt-2* probe consisted of a 2.1 kb HindIII containing the *cyt-2*$^+$ gene (Drygas, 1989). The 2.58 kb SalI fragment of pSV50 (Fig. 4) was used as the *tub-2* probe. The exposure time for both the *cya-5* and *cyt-2* probes was 144 hours (6 days). The blot hybridized with the *tub-2* probe was exposed for 24 hours. Position of molecular weight markers (in kb) are indicated at left.

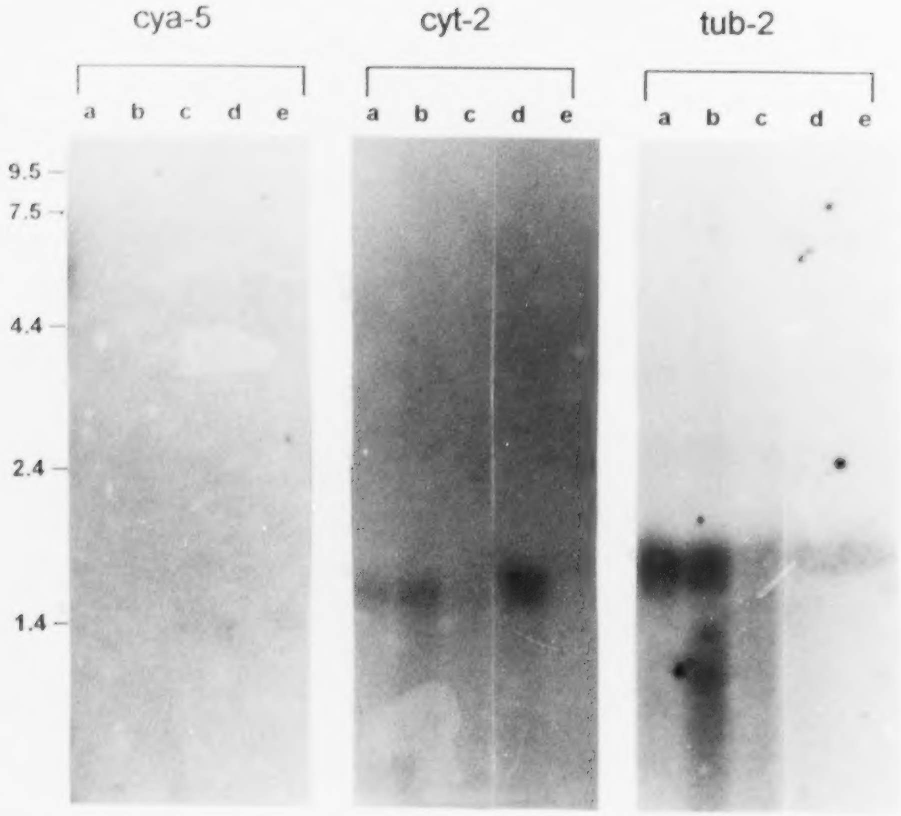
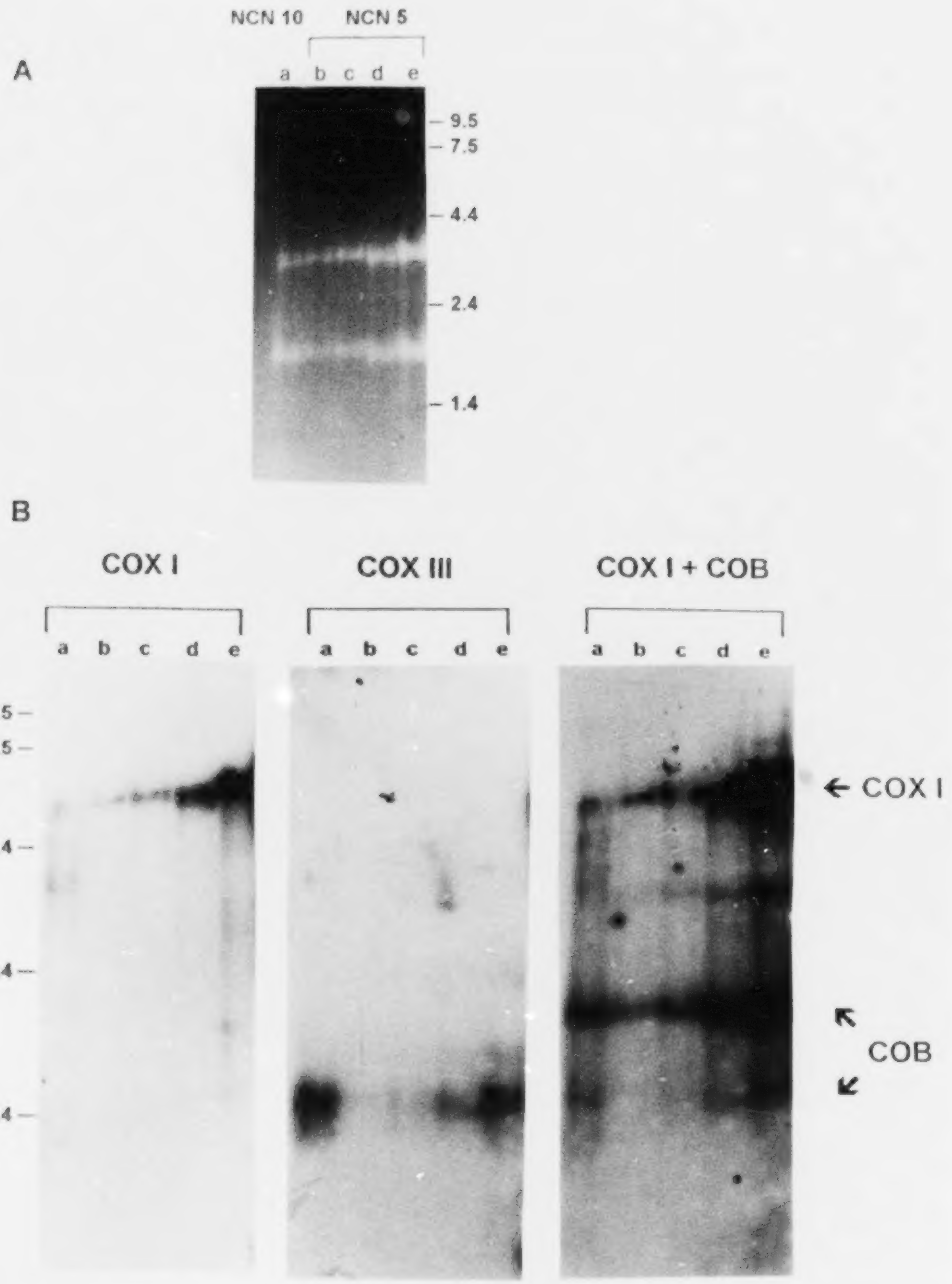

cya-5
cyt-2
tub-2
a b c d e
a b c d e
a b c d e
9.5
7.5
4.4
2.4
1.4

Figure 15. Northern analysis of mitochondrial RNA from *cya-5* and wild-type strains.

A. Ethidium bromide stained NCN5 and NCN10 mitochondrial RNA blotted onto a nylon membrane. RNA was separated on a 1% agarose gel. Lane a: 0.5 µg of NCN10 mitochondrial RNA. Lanes b-e: 0.2, 0.4, 0.7 and 1.4 µg, respectively, of NCN5 mitochondrial RNA. B. Blot shown in (A) probed with: (COX I), the HindIII 7c fragment of mtDNA containing the COX I gene (Burger et al., 1985); (COX III), the Pst1-1 fragment of mtDNA containing the COX III gene (Browning and RajBhandary, 1982); and (COX I + COB), the EcoRI-3 fragment of mtDNA containing the COX I, COB and $tRNA^{cys}$ genes (Burger et al., 1985). The two COB signals represent fully spliced COB transcripts that differ at the 5' untranslated ends (Kennell and Lambowitz, 1989). The smaller transcript (1.6 kb) appears to be initiated from a site approximately 500 nucleotides downstream of the initiation site for the larger transcript, but it is not known whether the smaller transcript is translated (Kennell and Lambowitz, 1989).


A
NCN 10
NCN 5
a b c d e
9.5
7.5
4.4
2.4
1.4
B
COX I
COX III
COX I + COB
a b c d e
a b c d e
a b c d e
9.5
7.5
4.4
2.4
1.4
COX I
COB

Bibliography

Ackerman, S.H., Martin, J., and Tzagoloff, A. 1992. Characterization of *ATP11* and detection of the encoded protein in mitochondria of *Saccharomyces cerevisiae*. J. Biol. Chem. 267: 7386-7394.

Ackerman, S.H., and Tzagoloff, A. 1990a. *ATP10*, a yeast nuclear gene required for the assembly of the mitochondrial F_1-F_0 complex. J. Biol. Chem. 265: 9952-9959.

Ackerman, S.H., and Tzagoloff, A. 1990b. Identification of two nuclear genes (*ATP11, ATP12*) required for assembly of the yeast F1-ATPase. Proc. Natl. Acad. Sci. USA 87: 4986-4990.

Agsteribbe, E., and Hartog, M. 1987. Processing of precursor RNAs from mitochondria of *Neurospora crassa*. Nucleic Acids Res. 15: 7249-7263.

Akins, R.A., and Lambowitz, A.M. 1984. The *[poky]* mutant of *Neurospora* contains a 4-base-pair deletion at the 5' end of the mitochondrial small rRNA. Proc. Natl. Acad. Sci. USA 81: 3791-3795.

Akins, R.A., and Lambowitz, A.M. 1985. General method for cloning *Neurospora crassa* nuclear genes by complementation of mutants. Mol. Cell. Biol. 5: 2272-2278.

Akins, R.A., and Lambowitz, A.M. 1987. A protein required for splicing Group I introns in *Neurospora* mitochondria is mitochondrial tyrosyl-tRNA synthetase or a derivative thereof. Cell 50: 331-345.

Altamura, N., Groudinsky, O., Dujardin, G., and Slonimski, P.P. 1992. *NAM7* nuclear gene encodes a novel member of a family of helicases with a Zn-ligand motif and is involved in mitochondrial functions in *Saccharomyces cerevisiae*. J. Mol. Biol. 224: 575-587.

Alton, N.K., Buxton, F., Patel, V., Giles, N.H., and Vapnek, D. 1982. 5' untranslated sequences of two structural genes in the *qa* gene cluster of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 79: 1955-1959.

Anderson, S., Bankier, A.T., Barrell, B.G., de Bruijn, M.H.L., Coulson, A.R., Drouin, J., Eperon, I.C., Nierlich, D.P., Roe, B.A., Sanger, F., Schreier, P.H., Smith, A.J.H., Staden, R., and Young, I.G. 1981. Sequence and organization of the human mitochondrial genome. Nature 290: 457-465.

Arends H., and Sebald, W. 1984. Nucleotide sequence of the cloned messenger-RNA and gene of the ADP/ATP carrier from *Neurospora crassa*. EMBO J. 3: 377-382.

Asch, D.K., and Kinsey, J.A. 1990. Relationship of vector insert size to homologous integration during transformation of *Neurospora crassa* with the cloned *am* (GDH) gene. Mol. Gen. Genet. 221: 37-43.

Asher, E.B., Groudinsky, O., Dujardin, G., Altamura, N., Kermorgant, M., and Slonimski, P.P. 1989. Novel class of nuclear genes involved in both mRNA splicing and protein synthesis in *Saccharomyces cerevisiae* mitochondria. Mol. Gen. Genet. 215: 517-528.

Attardi, G. 1985. Animal mitochondrial DNA: an extreme example of genetic economy. Int. Rev. Cytol. 93: 93-145.

Attardi, G., and Schatz, G. 1988. Biogenesis of mitochondria. Ann. Rev. Cell Biol. 4: 289-333.

Ballance, D.J. 1986. Sequences important for gene expression in filamentous fungi. Yeast 2: 229-236.

Bertrand, H., Bridge, P., Collins, R.A., Garriga, G., and Lambowitz, A.M. 1982. RNA splicing in *Neurospora* mitochondria. Characterization of new nuclear mutants with defects in the mitochondrial large rRNA. Cell 29: 517-526.

Bertrand, H., and Collins, R.A. 1978. A regulatory system controlling the production of cytochrome *aa3* in *Neurospora crassa.* Molec. Gen. Genet. 166: 1-13.

Bertrand, H., Nargang, F.E., Collins, R.A., and Zagozeski, C.A. 1977. Nuclear cytochrome deficient mutants of *Neurospora crassa*. Isolation, characterization and genetic mapping. Mol. Gen. Genet. 153: 247-257.

Bertrand, H., and Pittenger, T.H. 1969. Cytoplasmic mutants selected from continuously growing cultures of *Neurospora crassa*. Genetics 61: 643-659.

Bertrand, H., Szakacs, N.A., Nargang, F.E., Zagozeski, C.A., Collins, R.A., and Harrigan, J.C. 1976. The function of mitochondrial genes in *Neurospora crassa*. Can. J. Genet. Cytol. 18: 397-409.

Bertrand, H., and Werner, S. 1977. Deficiency of subunit two of cytochrome oxidase in the *[mi-3]* cytoplasmic mutant of *Neurospora crassa*. Eur. J. Biochem. 79: 599-606.

Bertrand, H., and Werner, S. 1979. Cytochrome *c* oxidase subunits in nuclear and extranuclear cytochrome-*aa3* deficient mutants of *Neurospora crassa*. Eur. J. Biochem. 98: 9-18.

Biswas, T.K., Baruch, T., and Getz, G.S. 1987. *In vitro* characterization of the yeast mitochondrial promoter using single-base substitution mutants. J. Biol. Chem. 262: 13690-13696.

Bogenhagen, D.F., Applegate, E.F., and Yoza, B.K. 1984. Identification of a promoter for transcription of the heavy strand of human mtDNA: *in vitro* transcription and deletion mutagenesis. Cell 36: 1105-1113.

Boguta, M., Dmochowska, A., Borsuk, P., Wrobel, K., Gargouri, A., Lazowska, J., Slonimski, P., Szczesniak, B., and Kruszewska, A. 1992. *NAM9* nuclear suppressor of mitochondrial ochre mutations in *Saccharomyces cerevisiae* codes for a protein homologous to S4 ribosomal proteins from chloroplasts, bacteria, and eucaryotes. Mol. Cell. Biol. 12: 402-412.

Bottorff, D.A. 1990. Characterization of the cytochrome *c* deficient mutant *cyc-1-12 of Neurospora crassa*. M.Sc. thesis, University of Alberta.

Bowman, S., Ackerman, S.H., Griffiths, D.E., and Tzagoloff, A. 1991. Characterization of *ATP12*, a yeast nuclear gene required for the assembly of the mitochondrial F1-ATPase. J. Biol. Chem. 266: 7517-7523.

Breitenberger, C.A., Browning, K.S., Alzner-DeWeerd, B., and RajBhandary, U.L. 1985. RNA processing in *Neurospora crassa* mitochondria: use of transfer RNA sequences as signals. EMBO J. 4: 185-195.

Breitenberger, C.A., and RajBhandary, U.L. 1985. Some highlights of mitochondrial research based on analysis of *Neurospora crassa* mitochondrial DNA. Trends Biochem. Sci. 10: 478-483.

Brown, T.A. 1987. The mitochondrial genomes of filamentous fungi. In "Gene structure in eukaryotic microbes". ed. Kinghorn, J. IRL Press. Oxford. pp. 141-162.

Browning, K.S., and RajBhandary, U.L. 1982. Cytochrome oxidase subunit III gene in *Neurospora crassa* mitochondria: location and sequence. J. Biol. Chem. 257: 5253-5256.

Brunori, M., Antonini, G., Malatesta, F., Sarti, P., and Wilson, M.T. 1987. Cytochrome *c* oxidase: subunit structure and proton pumping. Eur. J. Biochem. 169: 1-8.

Burger, G., Citterich, M.H., Nelson, M.A., Werner, S., and Macino, G. 1985. RNA processing in *Neurospora crassa* mitochondria: transfer RNAs punctuate a large precursor transcript. EMBO J. 4: 197-204.

Burger, G., Scriven, C., Machleidt, W., and Werner, S. 1982. Subunit 1 of cytochrome oxidase from *Neurospora crassa*: nucleotide sequence of the coding gene and partial amino acid sequence of the protein. EMBO J. 1: 1385-1391.

Burke, J.M., Breitenberger, C., Heckman, J.E., Dujon, B., and RajBhandary, U.L. 1984. Cytochrome *b* gene of *Neurospora crassa* mitochondria. J. Biol. Chem. 259: 504-511.

Butow, R.A., Farrelly, F., Zassenhaus, H.P., Hudspeth, M.E.S., Grossman, L.I., and Perlman, P.S. 1982. *var 1* determinant region of yeast mitochondrial DNA. In "Mitochondrial Genes". eds. Slonimski, P., Borst, P., and Attardi, G. Cold Spring Harbor Laboratory Press, New York. pp. 241-253.

Cabral, F., and Schatz, G. 1978. Identification of cytochrome *c* oxidase subunits in nuclear yeast mutants lacking the functional enzyme. J. Biol. Chem. 253: 4396-4401.

Capaldi, R.A. 1990. Structure and function of cytochrome *c* oxidase. Annu. Rev. Biochem. 59: 569-596.

Case, M.E. 1986. Genetical and molecular analyses of *qa-2* transformants in *Neurospora crassa*. Genetics 113: 569-587.

Case, M.E., Schweizer, M., Kushner, S.R., and Giles, N.H. 1979. Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc. Natl. Acad. Sci. USA 76: 5259-5263.

Casey, R.P., Thelen, M., Azzi, A. 1980. Dicyclohexylcarbodiimide binds specifically and covalently to cytochrome *c* oxidase while inhibiting its H^+-translocating activity. J. Biol. Chem. 255: 3994-4000.

Cech, T.R. 1990. Self-splicing of group I introns. Annu. Rev. Biochem. 59: 543-568.

Chang, D.D., and Clayton, D.A. 1984. Precise identification of individual promoters for transcription of each strand of human mitochondrial DNA. Cell 36: 635-643.

Chao, S., Sederoff, R.R., and Levings, C.S., III. 1983. Partial sequence of the 5S-18S gene region of the *maize* mitochondrial genome. Plant Physiol. 71: 190-193.

Chatton, B., Walter, P., Ebel, J.-P., Lacroute, F., Fasiolo, F. 1988. The yeast *VAS1* gene encodes both mitochondrial and cytoplasmic valyl-tRNA synthetases. J. Biol. Chem. 263: 52-57.

Chen, S.-Y., Ephrussi, B., et Hottinguer, H. 1950. Nature genetique des mutants a deficience respiratoire de la souche B-II de la levure de Boulangerie. Heredity 4: 337-351.

Cherniack, A.D., Garriga, G., Kittle, J.D., Jr., Akins, R.A., and Lambowitz, A.M. 1990. Function of *Neurospora* mitochondrial tyrosyl-tRNA synthetase in RNA splicing requires an idiosyncratic domain not found in other synthetases. Cell 62: 745-755.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., and Rutter, W.J. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. 1979. Biochemistry 18: 5294-5299.

Chow, C.M., Metzenberg, R.L., and RajBhandary, U.L. 1989. Nuclear gene for mitochondrial leucyl-tRNA synthetase of *Neurospora crassa:* isolation, sequence, chromosomal mapping, and evidence that the *leu-5* locus specifies structural information. Mol. Cell. Biol. 9: 4631-4644.

Christianson, T., Edwards, J.C., Mueller, D.M., Rabinowitz, M. 1983. Identification of a single transcriptional initiation site for the glutamic tRNA and *COB* genes in yeast mitochondria. Proc. Natl. Acad. Sci. USA 80: 5564-5568.

Christianson, T., and Rabinowitz, M. 1983. Identification of multiple transcriptional initiation sites on the yeast mitochondrial genome by *in vitro* capping with guanylyltransferase. J. Biol. Chem. 258: 14025-14033.

Clark-Walker, G.D. 1992. Evolution of mitochondrial genomes in fungi. Int. Rev. Cytol. 141: 89-127.

Clark-Walker, G.D., and Sriprakash, K.S. 1982. Size diversity and sequence rearrangements in mitochondrial DNAs from yeast. In "Mitochondrial Genes". eds. P. Slonimski, P. Borst and G. Attardi. Cold Spring Harbor Laboratory Press, N.Y. pp. 349-354.

Clayton, D.A. 1991. Replication and transcription of vertebrate mitochondrial DNA. Annu. Rev. Cell Biol. 7: 453-478.

Clayton, D.A. 1992. Transcription and replication of animal mitochondrial DNAs. Int. Rev. Cytol. 141: 217-232.

Collins, R.A., and Lambowitz, A.M. 1983. Structural variations and optional introns in the mitochondrial DNAs of *Neurospora* strains isolated from nature. Plasmid 9: 53-70.

Collins, R.A., and Lambowitz, A.M. 1985. RNA splicing in *Neurospora* mitochondria. Defective splicing of mitochondrial mRNA precursors in the nuclear mutant *cyt-18-1*. J. Mol. Biol. 184: 413-428.

Costanzo, M.C., and Fox, T.D. 1986. Product of *S. cerevisiae* nuclear gene *PET 494* activates translation of a specific mitochondrial mRNA. Mol. Cell. Biol. 6: 3694-3703.

Costanzo, M.C., and Fox, T.D. 1988. Specific translational activation by nuclear gene products occurs in the 5' untranslated leader of a yeast mitochondrial mRNA. Proc. Natl. Acad. Sci. USA. 85: 2677-2681.

Costanzo, M.C., and Fox, T.D. 1990. Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. Annu. Rev. Genet. 24: 91-113.

Costanzo, M.C., Seaver, E.C., and Fox, T.D. 1986. At least two nuclear gene products are specifically required for translation of a single yeast mitochondrial mRNA. EMBO J. 5: 3637-3641.

Costanzo, M.C., Seaver, E.C., and Fox, T.D. 1989. The *PET54* gene of *Saccharomyces cerevisiae*: Characterization of a nuclear gene encoding a mitochondrial translational activator and subcellular localization of its product. Genetics 122: 297-305.

Cummings, D.J. 1992. Mitochondrial genomes of the ciliates. Int. Rev. Cytol. 141: 1-64.

Davis, B.D., and Mingioli, E.S. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. 60: 17-28.

Davis, R.H., and de Serres, F.J. 1970. Genetic and microbiological research techniques for *Neurospora crassa*. Methods Enzymol. 17A: 79-143.

Decoster, E., Simon, M., Hatat, D., and Faye, G. 1990. The *MSS51* gene product is required for the translation of the COX I mRNA in yeast mitochondria. Mol. Gen. Genet. 224: 111-118.

De La Salle, H., Jacq, C., Slonimski, P.P. 1982. Critical sequences within mitochondrial introns: pleiotropic mRNA maturase and cis-dominant signals of the box intron controlling reductase and oxidase. Cell 28: 721-732.

Del Giudice, L., Massardo, D.R., Manna, F., and Wolf, K. 1986. Isolation and characterization of a conditional mutant in *Saccharomyces cerevisiae* producing rho^o petites at the non-permissive temperature. Curr. Genet. 11: 201-204.

Denis, M. 1986. Structure and function of cytochrome *c* oxidase. Biochime 68: 459-470.

de Vries, H., Haima, P., Brinker, M., and de Jonge, J.C. 1985. The *Neurospora* mitochondrial genome: the region coding for the polycistronic cytochrome oxidase subunit 1 transcript is preceded by a transfer RNA gene. FEBS Lett. 179: 337-342.

Dieckmann, C.L., Pape, L.K., and Tzagoloff, A. 1982. Identification and cloning of a yeast nuclear gene *(CBP1)* involved in expression of mitochondrial cytochrome *b*. Proc. Natl. Acad. Sci. USA. 79: 1805-1809.

Dieckmann, C.L., Koerner, T.J., and Tzagoloff, A. 1984. Assembly of the mitochondrial membrane system: *CBP1*, a yeast nuclear gene involved in 5' processing of cytochrome *b* pre-mRNA. J. Biol. Chem. 259: 4722-4731.

Dieckmann, C.L., and Mittelmeier, T.M. 1987. Nuclearly-encoded *CBP1* interacts with the 5' end of mitochondrial cytochrome *b* pre-mRNA. Curr. Genet. 12: 391-397.

Dieckmann, C.L., and Tzagoloff, A. 1985. Assembly of the mitochondrial membrane system: *CBP6*, a yeast nuclear gene necessary for the synthesis of cytochrome *b*. J. Biol. Chem. 260: 1513-1520.

Dobinson, K.F., Henderson, M., Kelly, R.L., Collins, R.A., and Lambowitz, A.M. 1989. Mutations in nuclear gene *cyt-4* of *Neurospora crassa* result in pleiotropic defects in processing and splicing of mitochondrial RNAs. Genetics 123: 97-108.

Dowhan, W., Bibus, C.R., and Schatz, G. 1985. The cytoplasmically-made subunit IV is necessary for assembly of cytochrome *c* oxidase in yeast. EMBO J. 4: 179-184.

Drygas, M.E. 1990. Elucidation of the *cyt-2-1* mutation of *Neurospora crassa*. Ph.D. thesis, University of Alberta.

Drygas, M.E., Lambowitz, A.M., and Nargang, F.E. 1989. Cloning and analysis of the *Neurospora crassa* gene for cytochrome *c* heme lyase. J. Biol. Chem. 264: 17897-17906.

Dujardin, G., Pajot, P., Groudinsky, O., and Slonimski, P.P. 1980. Long range control circuits within mitochondria and between nucleus and mitochondria. I. Methodology and phenomenology of suppressors. Mol. Gen. Genet. 179: 469-482.

Dujon, B. 1981. Mitochondrial genetics and functions. In "Molecular Biology of the yeast *Saccharomyces*. Life cycle and inheritance". eds. Strathern, J.N., Jones, E.W., and Broach, J.R. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y. pp. 505-635.

Ebner, E., Mason, T.L., and Schatz, G. 1973. Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae*. II. Effect of nuclear and extrachromosomal mutations on the formation of cytochrome *c* oxidase. J. Biol. Chem. 248: 5369-5378.

Edwards, D.L., Kwiecinski, F., and Horstmann, J. 1973. Selection of respiratory mutants of *Neurospora crassa*. J. Bacteriol. 114: 164-168.

Edwards, J.C., Levens, D., and Rabinowitz, M. 1982. Analysis of transcriptional initiation of yeast mitochondrial DNA in a homologous *in vitro* transcription system. Cell 31: 337-346.

Ephrussi, B., and Slonimski, P.P. 1950. La synthese adaptive des cytochromes chez la levure de boulangerie. Biochim. Biophys. Acta 6: 256-267.

Ephrussi, B., and Hottinguer, H. 1951. Cytoplasmic constituents of heredity: On an unstable cell state in yeast. Cold Spring Harbor Symp. Quant. Biol. 16: 75-85.

Falcone, C., Agostinelli, M., Frontali, L. 1983. Mitochondrial translation products during release from glucose repression in *Saccharomyces cerevisiae*. J. Bacteriol. 153: 1125-1132.

Faye, G., and Simon, M. 1983. Analysis of a yeast nuclear gene involved in the maturation of mitochondrial pre-messenger RNA of the cytochrome *c* oxidase subunit I. Cell 32: 77-87.

Fearon, K., and Mason, T.L. 1988. Structure and regulation of a nuclear gene in *Saccharomyces cerevisiae* that specifies *MRP7*, a protein of the large subunit of the mitochondrial ribosome. Mol. Cell. Biol. 8: 3636-3646.

Feinberg, A.P., and Vogelstein, B. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132: 6-13.

Feinberg, and Vogelstein, B. 1984. Addendum: "A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity". Anal. Biochem. 137: 266-267.

Forsbach, V., Pillar, T., Gottenof, T., and Rodel, G. 1989. Chromosomal localization and expression of *CBS1*, a translational activator of cytochrome *b* in yeast. Mol. Gen. Genet. 218: 57-63.

Forsburg, S.L., and Guarente, L. 1989. Communication between mitochondria and the nucleus in regulation of cytochrome genes in the yeast *Saccharomyces cerevisiae.* Annu. Rev. Cell Biol. 5: 153-180.

Fourney, R.M., Miyakoshi, J., Day III, R.S., and Paterson, M.C. 1988. Northern blotting: Efficient RNA staining and transfer. Focus 10: 5-7.

Fox, T.D. 1986. Nuclear gene products required for translation of specific mitochondrially coded mRNAs in yeast. Trends Genet 2: 97-100.

Freitag, H., Janes, M., and Neupert, W. 1982. Biosynthesis of mitochondrial porin and insertion into the outer mitochondrial membrane of *Neurospora crassa.* Eur. J. Biochem. 126: 197-202.

Frohman, M.A., Dush, M.K., and Martin, G.R. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA 85: 8998-9002.

Fu, Y.-H., J.V., Mannix, D.G., and Marzluf, G.A. 1989. *cys-3*, the positive-acting sulfur regulatory gene of *Neurosproa crassa*, encodes a protein with a putative leucine zipper DNA-binding element. Mol. Cell. Biol. 9: 1120-1127.

Fu, Y.-H., and Marzluf, G.A. 1990. *nit-2*, the major nitrogen regulatory gene of *Neurospora crassa*, encodes a protein with a putative zinc finger DNA-binding domain. Mol. Cell. Biol. 10: 1056-1065.

Gampel, A., and Cech, T.R. 1991. Binding of the *CBP2* protein to a yeast mitochondrial group I intron requires the catalytic core of the RNA. Genes Dev. 5: 1870-1880.

Gampel, A., Nishikimi, M., and Tzagoloff, A. 1989. *CBP2* protein promotes *in vitro* excision of a yeast mitochondrial group 1 intron. Mol. Cell. Biol. 9: 5424-5433.

Garriga, G., Bertrand, H., and Lambowitz, A.M. 1984. RNA splicing in *Neurospora* mitochondria: nuclear mutants defective in both splicing and 3' end synthesis of the large rRNA. Cell 36: 623-634.

Garriga, G., and Lambowitz, A.M. 1984. RNA splicing in *Neurospora* mitochondria: self-splicing of a mitochondrial intron *in vitro*. Cell 38: 631-641.

Garriga, G., and Lambowitz, A.M. 1986. Protein-dependant splicing of group I inton in ribonucleoprotein particles and soluble fractions. Cell 46: 669-680.

Gavel, Y., and von Heijne, G. 1990. Cleavage-site motifs in mitochondrial targeting peptides. Prot. Eng. 4: 33-37.

Giles, N.H., Case, M.E., Baum, J., Geever, R., Huiet, L., Patel, V., and Tyler, B. 1985. Gene organization and regulation in the *qa* (quinic acid) gene cluster of *Neurospora crassa*. Microbiol. Rev. 49: 338-358.

Gray, M.W. 1989. Origin and evolution of mitochondrial DNA. Annu. Rev. Cell Biol. 5: 25-50.

Gray, M.W., and Spencer, D.F. 1981. Is wheat mitochondrial 5S ribosomal RNA prokaryotic in nature? Nucleic Acids Res. 9: 3523-3529.

Green, M.R., Grimm, M.F., Goewert, R.R., Collins, R.A., Cole, M.D., Lambowitz, A.M., Heckman, J.E., Yin, S., and RajBhandary, U.L. 1981. Transcripts and processing patterns for the ribosomal RNA and transfer RNA region of *Neurospora crassa* mitochondrial DNA. J. Biol. Chem. 256: 2027-2034.

Greenleaf, A.L., Kelly, J.L.,and Lehman, I.R. 1986. Yeast *RP041* gene product is required for transcription and maintenance of the mitochondrial genome. Proc. Natl. Acad. Sci. USA 83: 3391-3394.

Grivell, L.A. 1989. Nucleo-mitochondrial interactions in yeast mitochondrial biogenesis. Eur. J. Biochem. 182: 477-493.

Grohmann, L., Graack, H.R., Kitakawa, M. 1989. Molecular cloning of the nuclear gene for mitochondrial ribosomal protein *YmL31* from *Saccharomyces cerevisiae*. Eur. J. Biochem. 183: 155-160.

Groudinsky, O., Dujardin, G., and Slonimski, P.P. 1981. Long range control circuits within mitochondria and between nucleus and mitochondria. II. Genetic and biochemical analysis of suppressors which selectively alleviate the mitochondrial intron mutations. Mol. Gen. Genet. 184: 493-503.

Guo, Q., Akins, R.A., Garriga, G., and Lambowitz, A.M. 1991. Structural analysis of the *Neurospora* mitochondrial large rRNA intron and construction of a mini-intron that shows protein-dependent splicing. J. Biol. Chem. 266:1809-1819.

Gurr, S.J., Unkles, S.E., and Kinghorn, J.R. 1987. The structure and organization of nuclear genes of filamentous fungi. In "Gene structure in eukaryotic microbes". ed. Kinghorn, J.R. IRL Press. Oxford. pp. 93-139.

Haffter, P., McMullin, T.W., and Fox, T.D. 1990. A genetic link between an mRNA-specific translational activator and the translation system in yeast mitochondria. Genetics 125: 495-503.

Haffter, P., McMullin, T.W., and Fox, T.D. 1991. Functional interactions among two yeast mitochondrial ribosomal proteins and an mRNA-specific translational activator. Genetics 127: 319-326.

Hanson, M.R., and Folkerts, O. 1992. Structure and function of the higher plant mitochondrial genome. Int. Rev. Cytol. 141: 129-172.

Hartl, F.-U., Pfanner, N., Nicholson, D.W., and Neupert, W. 1989. Mitochondrial protein import. Biochim. Biophys. Acta 998: 1-45.

Herbert, C.J.M., Labouesse, M., Dujardin, G., and Slonimski, P.P. 1988. The *NAM2* proteins of *S. cerevisiae* and *S. douglasii* are mitochondrial leucyl-tRNA synthetases, and are involved in mRNA splicing. EMBO J. 7: 473-483.

Hill, J., McGraw, P., and Tzagoloff, A. 1985. A mutation in yeast mitochondrial DNA results in a precise excision of the terminal intron of the cytochrome *b* gene. J. Biol. Chem. 260: 3235-3238.

Hixson, J.E., and Clayton, D.A. 1985. Initiation of transcription from each of the two human mitochondrial promoters requires unique nucleotides at the transcriptional start sites. Proc. Natl. Acad. Sci. USA 82: 2660-2664.

Hodgman, T.C. 1988. A new superfamily of replicative proteins. Nature 333: 22-23.

Hsiao, K. 1991. A fast and simple procedure for sequencing double stranded DNA with Sequenase. Nucleic Acids Res. 19: 2787.

Huiet, L., and Giles, N.H. 1986. The *qa* repressor gene of *Neurospora crassa:* Wild-type and mutant nucleotide sequence. Proc. Natl. Acad. Sci. USA 83: 3381-3385.

Hultmark, D., Klemenz, R., and Gehring, W. 1986. Translational and transcriptional control elements in the untranslated leader of the heat shock gene *hsp22*. Cell 44: 429-438.

Hunt, T. 1985. False starts in translational control of gene expression. Nature 316: 580-581.

Jang, S.H., and Jaehning, J.A. 1991. The yeast mitochondrial RNA polymerase specificity factor, *MTF1*, is similar to bacterial sigma factors. J. Biol. Chem. 266: 22671-22677.

Kadenbach, B., and Merle, P. 1981. On the function of multiple subunits of cytochrome *c* oxidase from higher eukaryotes. FEBS Lett. 135: 1-11.

Katz, R.A. Cullen, B. R. Malavarca, R., and Skalka, A.M. 1986. Role of the avian retrovirus mRNA leader in expression: evidence for novel translational control. Mol. Cell. Biol. 6: 372-379.

Kennell, J.C., and Lambowitz, A.M. 1989. Development of an *in vitro* transcription system for *Neurospora crassa* mitochondrial DNA and identification of transcription initiation sites. Mol. Cell. Biol. 9: 3603-3613.

Kinnaird, J.H., and Fincham, J.R.S. 1983. The complete nucleotide sequence of the *Neurospora crassa am* (NADP-specific glutamate dehydrogenase) gene. Gene 26: 253-260.

Kirk, M.M., and Kirk, D.L. 1985. Translational regulation of protein synthesis, in response to light, at a critical stage of *Volvox* development. Cell 41: 419-428.

Klemenz, R., Hultmark, D., and Gehring, W.J. 1985. Selective translation of heat shock mRNA in *Drosophila melanogaster* depends on sequence information in the leader. EMBO J. 4: 2053-2060.

Kloeckener-Gruissem, B., McEwen, J.E., and Poyton, R.O. 1988. Identification of a third nuclear protein-coding gene required specifically for post-transcriptional expression of the mitochondrial COX3 gene in *Saccharomyces cerevisiae*. J. Bacteriol. 170: 1399-1402.

Kozak, M. 1983. Comparisons of initiation of protein synthesis in procaryotes, eucaryotes and organelles. Microbiol. Rev. 47: 1-45.

Kozak, M. 1989. The scanning model for translation: an update. J. Cell Biol. 108: 229-241.

Kreader, C.A., Langer, C.S., and Heckman, J.E. 1989. A mitochondrial protein from *Neurospora crassa* detected both on ribosomes and in membrane fractions. J. Biol. Chem. 264: 317-327.

Krieke, J., Schulze, M., Pillar, T., Koetre, A., and Rödel, G. 1986. Cloning of a nuclear gene *MRS1* involved in the excision of a single group I intron (bI3) from the mitochondrial *COB* trancript in *S. cerevisiae*. Curr. Genet. 11: 185-191.

Krummeck, G., and Rodel, G. 1990. Yeast *SCO1* protein is required for a post-translational step in the accumulation of mitochondrial cytochrome *c* oxidase subunits 1 and 2. Curr. Genet. 18: 13-15.

Kubelik, A.R., Kennell, J.C., Akins, R.A., and Lambowitz, A.M. 1990. Identification of *Neurospora* mitochondrial promoters and analysis of synthesis of the mitochondrial small rRNA in wild-type and the promoter mutant *[poky]*. J. Biol. Chem. 265: 4515-4526.

Kubelik, A.R., Turcq, B., and Lambowitz, A.M. 1991. The *Neurospora crassa cyt-20* gene encodes cytosolic and mitochondrial valyl-tRNA synthetases and may have a second function in addition to protein synthesis. Mol. Cell. Biol. 11: 4022-4035.

Kuiper, M.T.R., Akins, R.A., Holtrop, M., de Vries H., and Lambowitz, A.M. 1988. Isolation and analysis of the *Neurospora crassa cyt-21* gene. A nuclear gene encoding a mitochondrial ribosomal protein. J. Biol. Chem. 263: 2840-2847.

Labouesse, M. 1990. The yeast mitochondrial leucyl-tRNA synthetase is a splicing factor for the excision of several group I introns. Mol. Gen. Genet. 224: 209-221.

Labouesse, M., Netter, P., and Schroeder, P. 1984. Molecular basis of the box effect: a maturase deficiency leading to the absence of splicing of two introns located in two split genes of yeast mitochondrial DNA. Eur. J. Biochem. 144: 85-93.

Labouesse, M., Dujardin, G., and Slonimski, P.P. 1985. The yeast nuclear gene *NAM2* is essential for the mitochondrial DNA integrity and can cure a mitochondrial RNA-maturase deficiency. Cell 41: 133-143.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680-685.

Lambowitz, A.M. 1979. Preparation and analysis of mitochondrial ribosomes. Methods Enzymol. 59: 421-433.

Lambowitz, A.M., and Slayman, C.W. 1971. Cyanide-resistant respiraton in *Neurospora crassa*. J. Bacteriol. 108:1087-1096

LaPolla, R.J., and Lambowitz, A.M. (1981). Mitochondrial ribosome assembly in *Neurospora crassa*. J. Biol. Chem. 256: 7064-7067.

Lazowska, J., Jacq, C., and Slonimski, P.P. 1980. Sequence of introns and flanking exons in wild-type and box3 mutants of cytochrome *b* reveals an interlaced splicing protein coded by an intron. Cell 22: 333-348.

Lazowska, J., Claisse, M., Gargouri, A., Kotylak, Z., Spyrydakis, A., and Slonimski, P. 1989. Protein encoded by the third intron of cytochrome *b* gene in *Saccharomyces cerevisiae* is an mRNA maturase. Analysis of mitochondrial mutants, RNA transcripts, proteins and evolutionary relationships. J. Mol. Biol. 205: 275-289.

Legerton, T., and Yanofsky, C. 1985. Cloning and characterization of the multifunctional *his-3* gene of *Neurospora crassa*. Gene 39: 129-140.

Lemire, E.G., and Nargang, F.E. 1986. A missense mutation in the *oxi-3* gene of the *[mi-3]* extranuclear mutant of *Neurospora crassa*. J. Biol. Chem. 261: 5610-5615.

Levens, D., Ticho, B., Ackerman, E., Rabinowitz, M. 1981. Transcriptional initiation and 5' termini of yeast mitochondrial RNA. J. Biol. Chem. 256: 5226-5232.

Lisowsky, T. 1990. Molecular analysis of the mitochondrial transcription factor *mtf2* of *Saccharomyces cerevisiae*. Mol. Gen. Genet. 220: 186-190.

Logan, J., and Shenk, T. 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. Proc. Natl. Acad. Sci. USA 81: 3655-3659.

Lonsdale, D.M., Brears, T., Hodge, T.P., Melville, S.E., and Rottmann, W.H. 1988. The plant mitochondrial genome: homologous recombination as a mechanism for generating heterogeneity. Phil. Trans. R. Soc. Lond. B 319: 149-163.

Luck, D.J.L. 1963. Genesis of mitochondria in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 49: 233-240.

Luck, D.J.L. 1965. Formation of mitochondria in *Neurospora crassa*. J. Cell Biol. 24: 461-470.

Luck, D.J.L., and Reich, E. 1964. DNA in mitochondria of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 52: 931-938.

Majumder, A.L., Akins, R.A., Wilkinson, J.G., Kelley, R.L., Snook, A.J., and Lambowitz, A.M. 1989. Involvement of tyrosyl-tRNA synthetase in splicing of group I introns in mitochondria: Biochemical and immunological analyses of splicing activity. Mol. Cell. Biol. 9: 2089-2104

Malmstrom, B.G., Nilsson, T. 1988. Intermediate states and electron gating in proton-pumping cytochrome *c* oxidase. Ann. NY. Acad. Sci. 550: 177-184.

Mandel, M., and Higa, A. 1970. Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159-162.

Maniatis, T., Fritsch, E.F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Mann, B.J., Bowman, B.J., Grotelueschen, J., and Metzenberg, R.L. 1989. Nucleotide sequence of *pho-4*$^+$, encoding a phosphate-repressible phosphate permease of *Neurospora crassa*. Gene 83: 281-289.

Mannella, C.A., Collins, R.A., Green, M.R., and Lambowitz, A.M. 1979. Defective splicing of mitochondrial rRNA in cytochrome-deficient nuclear mutants of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 76: 2635-2639.

Marykwas, D.L., and Fox, T.D. 1989. Control of the *Saccharomyces cerevisiae* regulatory gene *PET494*: transcriptional repression by glucose and translational induction by oxygen. Mol. Cell. Biol. 9: 484-491.

Matsushita, Y., Kitakawa, M., Isono, K. 1989. Cloning and analysis of the nuclear genes for two mitochondrial ribosomal proteins in yeast. Mol. Gen. Genet. 219: 119-124.

McEwen, J.E., Cameron, V.L., and Poyton, R.O. 1985. Rapid method for isolation and screening of cytochrome *c* oxidase deficient mutants of *Saccharomyces cerevisiae*. J. Bacteriol. 161: 831-835.

McGarry, T.J., and Lindquist, S. 1985. The preferential translation of *Drosophila hsp70* mRNA requires sequences in the untranslated leader. Cell 42: 903-911.

McGraw, P. and Tzagoloff, A. 1983. Assembly of the mitochondrial membrane system: Characterization of a yeast nuclear gene involved in the processing of the cytochrome *b* pre-mRNA. J. Biol. Chem. 258: 9459-9468.

McMullin, T.W., Haffter, P., and Fox, T.D. 1990. A novel small subunit ribosomal protein of yeast mitochondria that interacts functionally with an mRNA-specific translational activator. Mol. Cell. Biol. 10: 4590-4595.

Messing, J. 1983. New M13 vectors for cloning. Methods Enzymol. 101C: 20-78.

Michaelis, U., Korte, A., and Rodel, G. 1991. Association of cytochrome *b* translational activator proteins with the mitochondrial membrane: implications for cytochrome *b* expression in yeast. Mol. Gen. Genet. 230: 177-185.

Michaelis, U., and Rodel, G. 1990. Identification of *CBS2* as a mitochondrial protein in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 223: 394-400.

Mieszczak, M., Kozlowski, M., Zagorski, W. 1988. Protein composition of *Saccharomyces cerevisiae* mitochondrial ribosomes. Acta Biocim. Pol. 35: 105-118.

Mitchell, M.B., and Mitchell, H.K. 1952. A case of "maternal" inheritance in *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 38: 442-449.

Mitchell, M.B., Mitchell, H.K., and Tissieres, A. 1953. Mendelian and Non-Mendelian factors affecting the cytochrome system in *Neurospora crassa*. Proc. Nat. Acad. Sci. USA 39: 606-613.

Mitchell, P. 1976. Possible molecular mechanisms of the protonmotive function of cytochrome systems. J. Theoret. Biol. 62: 327-367.

Mitchell, P., Mitchell, R., Moody, A.J., West, I.C., Baum, H., and Wrigglesworth, J.M. 1985. Chemiosmotic coupling in cytochrome oxidase: Possible protonmotive O loop and O cycle mechanisms. FEBS Lett. 188: 1-7.

Mohr, G., Zhang, A., Gianelos, J.A., Belfort, M., and Lambowitz, A.M. 1992. The *Neurospora CYT-18* protein suppresses defects in the phage T4 *td* intron by stabilizing the catalytically active structure of the intron core. Cell 69: 483-494.

Montague, P. 1987. Comparisons of the molecular structure between mammalian and lower eukaryotic genes. In "Gene structure in eukaryotic microbes". ed. Kinghorn, J.R. IRL Press. Oxford. pp. 263-285.

Mounolou, J.C., Jakob, H., and Slonimski, P.P. 1966. Mitochondrial DNA from "petite" mutants: Specific changes of buoyant density corresponding to different cytoplasmic mutations. Biochem. Biophys. Res. Communic. 24: 218-223.

Mueller, D.M., Biswas, T.K., Backer, J., Edwards, J.C., Rabinowitz, M., and Getz, G.S. 1987. Temperature sensitive pet mutants in yeast *Saccharomyces cerevisiae* that lose mitochondrial RNA. Curr. Genet. 11: 359-367.

Mueller, D.M., and Fox, T.D. 1984. Moleculear cloning and genetic mapping of the *pet 494* gene of *Saccharomyces cerevisiae*. Mol. Gen. Genet. 195: 275-280.

Mueller, P.P., Harashima, S., and Hinnebusch, A.G. 1987. A segment of *GCN4* mRNA containing the upstream AUG codons confers translational control upon a heterologous yeast transcript. Proc. Natl. Acad. Sci. USA 84: 2863-2867.

Mulero, J.J., and Fox, T.D. 1993. *PET111* acts in the 5'-leader of the *Saccharomyces cerevisiae* mitochondrial COX2 mRNA to promote its translation. Genetics 133: 509-516.

Muller, P.P., Reif, M.K., Zonghou, S., Sengstag, C., Mason, T.L., and Fox, T.D. 1984. A nuclear mutation that post-transcriptionally blocks accumulation of a yeast mitochondrial gene product can be suppressed by a mitochondrial gene rearrangement. J. Mol. Biol. 175: 431-452.

Mulligan, R.M., and Walbot, V. 1986. Gene expression and recombination in plant mitochondrial genomes. Trends Genet. 2: 263-266.

Munger, K., Germann, U.A., and Lerch, K. 1985. Isolation and structural organization of the *Neurospora crassa* copper metallothionein gene. EMBO J. 4: 2665-2668

Myers, A.M., Crivellone, M.D., and Tzagoloff, A. 1987. Assembly of mitochondrial membrane system: *MRP1* and *MRP2*, two yeast nuclear genes coding for mitochondrial ribosomal proteins. J. Biol. Chem. 262: 3388-3397.

Myers, A.M., Pape, L.K., and Tzagoloff, A. 1985. Mitochondrial protein synthesis is required for maintenance of intact mitochondrial genomes in *Saccharomyces cerevisiae*. EMBO J. 4: 2087-2092.

Nargang, F.E., Bertrand, H., and Werner, S. 1978. A nuclear mutant mutant of *Neurospora crassa* lacking subunit 1 of cytochrome *c* oxidase. J. Biol. Chem. 253: 6364-6369.

Natsoulis, G., Hilger, F., Fink, G.R. 1986. The *HTS1* gene encodes both the cytoplasmic and mitochondrial histidine-tRNA synthetases of *S. cerevisiae*. Cell 46: 235-243.

Nehls, U., Friedrich, T., Schmiede, A., Ohnishi, T., and Weiss, H. 1992. Characterization of assembly intermediates of NADH: ubiquinone oxidoreductase (complex I) accumulated in *Neurospora* mitochondria by gene disruption. J. Mol. Biol. 227: 1032-1042.

Newbury, S.F., Glazebrook, J.A., and Radford, A. 1986. Sequence analysis of the *pyr-4* (orotidine 5'-P decarboxylase) gene of *Neurospora crassa*. Gene 43: 51-58.

Ohmen, J.D., Kloeckener-Gruissem, B., McEwen, J.E. 1988. Molecular cloning and nucleotide sequence of the nuclear *PET122* gene required for expression of the mitochondrial COX3 gene in *S. cerevisiae*. Nucleic Acids Res. 16: 10783-10802.

Ojala, D., Montoya, J., and Attardi, G. 1981. The tRNA punctuation model of RNA processing in human mitochondria. Nature 290: 470-474.

Okimoto, R., Macfarlane, J.L., Clary, D.O., and Wolstenholme, D.R. 1992. The mitochondrial genomes of two nematodes, *Caenorhabditis elegans* and *Ascaris suum*. Genetics 130: 471-498.

Orbach, M.J., Porro, E.B., and Yanofsky, C. 1986. Cloning and characterization of the gene for ß-tubulin from a benomyl resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. Mol. Cell. Biol. 6: 2452-2461.

Osinga, K.A., De Vries, E., Van der Horst, G., and Tabak, H.F. 1984. Processing of yeast mitochondrial messenger RNAs at a conserved dodecamer sequence. EMBO J. 3: 829-834.

Paietta, J.V., Akins, R.A., Lambowitz, A.M., Marzluf, G.A. 1987. Molecular cloning and characterization of the *cys-3* regulatory gene of *Neurospora crassa*. Mol. Cell. Biol. 7: 2506-2511.

Paluh, J.L., Orbach, M.J., Legerton, T.L., and Yanofsky, C. 1988. The cross-pathway control gene of *Neurospora crassa*, *cpc-1*, encodes a protein similar to *GCN4* of yeast and the DNA-binding domain of the oncogene v-jun-encoded protein. Proc. Natl. Acad. Sci. USA 85: 3728-3732.

Partaledis, J.A., and Mason, T.L. 1988. Structure and regulation of a nuclear gene in *Saccharomyces cerevisiae* that specifies *MRP13*, a protein of the small subunit of the mitochondrial ribosome. Mol. Cell. Biol. 8: 3647-3660.

Perkins, D.D., Radford, A., Newmeyer, D., and Bjorkman, M. 1982. Chromosomal loci of *Neurospora crassa*. Microbiol. Rev. 46: 2444-2448.

Perlman, P.S., and Butow, R.A. 1989. Mobile introns and intron-encoded proteins. Science 246: 1106-1109.

Pittenger, T.H., and West, D.J. 1979. Isolation and characterization of temperature-sensitive respiratory mutants of *Neurospora crassa*. Genetics 93: 539-555.

Poutre, C.G., and Fox, T.D. 1987. *PET111*, a *Saccharomyces cerevisiae* nuclear gene required for translation of the mitochondrial mRNA encoding cytochrome *c* oxidase subunit II. Genetics 115: 637-647

Power, S.D., Lochrie, M.A., Patterson, T.E., and Poyton, R.O. 1984. The nuclear-coded subunits of yeast cytochrome *c* oxidase. J. Biol. Chem. 259: 6564-6560.

Pratje, E., and Guiard, B. 1986. One nuclear gene controls the removal of transient pre-sequences from two yeast proteins: one encoded by the nuclear the other by the mitochondrial genome. EMBO J. 5: 1313-1317.

Pratje, E., Mannhaupt, G., Michaelis, G., and Beyreuther, K. 1983. A nuclear mutation prevents processing of a mitochondrially encoded membrane protein in *Saccharomyces cerevisiae*. EMBO J. 2: 1049-1054.

Pritchard, A.E., Seilhamer, J.J., Cummings, D.J. 1986. *Paramecium* mitochondrial DNA sequences and RNA transcripts for cytochrome oxidase subunit I, URFI, and three ORFs adjacent to the replication origin. Gene 44: 243-253.

Rao, C.D., Pech, M., Robbins, K.C., and Aaronson, S.A. 1988. The 5' untranslated sequence of the c-*sis*/platelet-derived growth factor 2 transcript is a potent translation inhibitor. Mol. Cell. Biol. 8: 284-292.

Roberts, A.N., Berlin, V., Hager, K.M., and Yanofsky, C. 1988. Molecular analysis of a *Neurospora crassa* gene expressed during conidiation. Mol. Cell. Biol. 8: 2411-2418.

Rodel, G. 1986. Two yeast nuclear genes, *CBS1* and *CBS2*, are required for translation of mitochondrial transcripts bearing the 5' untranslated *COB* leader. Curr. Genet. 11: 41-45.

Rodel, G., and Fox, T.D. 1987. The yeast nuclear gene *CBS1* is required for translation of mitochondrial mRNAs bearing the *cob* 5' untranslated leader. Mol. Gen. Genet. 206: 45-50.

Rodel, G., Korte, A., Kaudewitz, F. 1985. Mitochondrial suppression of a yeast nuclear mutation which affects the translation of the mitochondrial apocytochrome *b* transcript. Curr. Genet. 9: 641-648.

Rutledge, B.J. 1984. Molecular characterization of the *qa-4* gene of *Neurospora crassa*. Gene 32: 275-287.

Sachs, M.S., Bertrand, H., Metzenberg, R., and RajBhandary, U.L. 1989. Cytochrome oxidase subunit V gene of *Neurospora crassa:* DNA sequences, chromosomal mapping, and evidence that the *cya-4* locus specifies the structural gene for subunit V. Mol. Cell. Biol. 9: 566-577.

Sanger, F., Nicklen S., and Coulson, A.R. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.

Schatz, G., Haslbrunner, E., and Tuppy, H. 1964. Deoxyribonucleic acid associated with yeast mitochondria. Biochem. Biophys. Res. Communic. 15: 127-132.

Schechtman, M.G., and Yanofsky, C. 1983. Structure of the trifunctional *trp-1* gene from *Neurospora crassa* and its aberrant expression in *Escherichia coli*. J. Mol. Appl. Genetics 2: 83-99.

Schinkel, A.H., Groot Koerkamp, M.J.A., Stuiver, M.H., Van der Horst, G.T.J., and Tabak, H.F. 1987a. Effect of point mutations on *in vitro* transcription from the promoter for the large ribosomal RNA gene of yeast mitochondria. Nucleic Acids Res. 15: 5597-5612.

Schinkel, A.H., Groot Koerkamp, M.J.A., and Tabak, H.F. 1988. Mitochondrial RNA polymerase of *Saccharomyces cerevisiae*: composition and mechanism of promoter recognition. EMBO J. 7: 3255-3262.

Schinkel, A.H., Groot Koerkamp, M.J.A., Touw, E.P.W., Tabak, H.F. 1987b. Specificity factor of yeast mitochondrial RNA polymerase. J. Biol. Chem. 262: 12785-12791.

Schinkel, A.H., and Tabak, H.F. 1989. Mitochondrial RNA polymerase: dual role in transcription and replication. Trends Genet. 5: 149-154.

Schneider, H., Sollner, T., Dietmeier, K., Eckerskorn, C., Lottspeich, F., Trulzsch, B., Neupert, W., Pfanner, N. 1991. Targeting of the master receptor *MOM19* to mitochondria. Science 254: 1659-1662.

Schulze, M., and Rodel, G. 1988. *SCO1*, a yeast nuclear gene essential for accumulation of mitochondrial cytochrome *c* oxidase subunit 2. Mol. Gen. Genet. 211: 492-498.

Schulze, M., and Rodel, G. 1989. Accumulation of the cytochrome *c* oxidase subunits 1 and 2 in yeast requires a mitochondrial membrane-associated protein, encoded by the nuclear *SCO1* gene. Mol. Gen. Genet. 216: 37-43.

Schweizer, M., Case, M.E., Dykstra, C.C., Giles, N.H., and Kushner, S.R. 1981. Identification and characterization of recombinant plasmids carrying the complete *qa* gene cluster from *Neurospora crassa* including the *qa-1*$^+$ regulatory gene. Proc. Natl. Acad. Sci. USA 78: 5086-5090.

Sederoff, R.R. 1984. Structural variation in mitochondrial DNA. Adv. Genet. 22: 1-108.

Seraphin, B., Simon, M., Boulet, A., and Faye, G. 1989. Mitochondrial splicing requires a protein from a novel helicase family. Nature (London) 337: 84-87.

Simon, M., and Faye, G. 1984. Steps in processing of the mitochondrial cytochrome oxidase subunit I pre-mRNA affected by a nuclear mutation in yeast. Proc. Natl. Acad. Sci. 81: 8-12.

Snyder, M., Fraser, A.R., LaRoche, J., Gartner-Kepkay, K.E., Zouros, E. 1987. Atypical mitochondrial DNA from the deep-sea scallop *Placopecten megellanicus*. Proc. Natl. Acad. Sci. USA 84: 7595-7599.

Southern, E. 1979. Gel electrophoresis of restriction fragments. Methods Enzymol. 68: 152-176.

Strick, C.A., and Fox, T.D. 1987. *Saccharomyces cerevisiae* positive regulatory gene *PET111* encodes a mitochondrial protein that is translated from an mRNA with a long 5' leader. Mol. Cell. Biol. 7: 2728-2734.

Stuart, K., and Feagin, J.E. 1992. Mitochondrial DNA of kinetoplastids. Int. Rev. Cytol. 141: 65-88.

Stuart, R.A., Neupert, W., and Tropschug, M. 1987. Deficiency in mRNA splicing in a cytochrome *c* mutant of *Neurospora crassa:* importance of carboxy terminus for import of apocytochrome *c* into mitochondria. EMBO J. 6: 2131-2137.

Suyama, Y., Fukuhara, H., Sor, F. 1985. A fine restriction map of the linear mitochondrial DNA of *Tetrahymena pyriformis:* genome size, map locations of rRNA and tRNA genes, terminal inversion repeat, and restriction site polymorphism. Curr. Genet. 9: 479-493.

Taanman, J.-W., and Capaldi, R.A. 1992. Purification of yeast cytochrome *c* oxidase with a subunit composition resembling the mammalian enzyme. J. Biol. Chem. 267: 22481-22485.

Thalenfeld, B.E., Bonitz, S.G., Nobrega, F.G., Macino, G., and Tzagoloff, A. 1983. *oli1* transcripts in wild-type and in a cytoplasmic "petite" mutant of yeast. J. Biol. Chem. 258: 14065-14068.

Turcq, B., Dobinson, K.F., Serizawa, N., and Lambowitz, A.M. 1992. A protein required for RNA processing and splicing in *Neurospora* mitochondria is related to gene products involved in cell cycle protein phosphatase functions. Proc. Natl. Acad. Sci. USA 89: 1676-1680.

Tzagoloff, A. 1982. Mitochondria. Plenum Press, New York.

Tzagoloff, A., and Dieckman, C.L. 1990. PET genes of *Saccharomyces cerevisiae*. Microbiol. Rev. 54: 211-225.

Tzagoloff, A., and Myers, A.M. 1986. Genetics of mitochondrial biogenesis. Ann. Rev. Biochem. 55: 249-285.

Tzamarias, D., Alexandraki D., and Thireos, G. 1986. Multiple cis-acting elements modulate the translational efficiency of *GCN4* mRNA in yeast. Proc. Natl. Acad. Sci. USA 83: 4849-4853.

Valencik, M.L., Kloeckener-Gruissem, B., Poyton, R.O., and McEwen, J.E. 1989. Disruption of the yeast nuclear *PET54* gene blocks excision of mitochondrial intron aI5B from pre-mRNA for cytochrome *c* oxidase subunit 1. EMBO J. 8: 3899-3904.

Valencik, M.L., and McEwen, J.E. 1991. Genetic evidence that different functional domains of the *PET54* gene product facilitate expression of the mitochondrial genes COX 1 and COX 3 in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 11: 2399-2405.

van der Horst, G., and Tabak, H.F. 1985. Self-splicing of yeast mitochondrial ribosomal and messenger RNA precursors. Cell 40: 759-766.

Vollmer, S.J., and Yanofsky, C. 1986. Efficient cloning of genes of *Neurospora crassa*. Proc. Natl. Acad. Sci. USA 83: 4869-4873.

Weber, E.R., and Dieckmann, C.L. 1990. Identification of the *CBP1* polypeptide in mitochondrial extracts from *Saccharomyces cerevisiae.* J. Biol. Chem. 265: 1594-1600.

Weiss-Brummer, B., Rodel, G., Schweyen, R.J., and Kaudewitz, F. 1982. Expression of the split gene *cob* in yeast: evidence for a precursor of a "maturase" protein translated from intron 4 and preceding exons. Cell 29: 527-536.

Werner, M., Feller, A., Messenguy, F., and Pierard, A. 1987. The leader peptide of yeast gene *CPA1* is essential for the translational repression of its expression. Cell 49: 805-813.

Wikstrom, M.K.F. 1977. Proton pump coupled to cytochrome *c* oxidase in mitochondria. Nature (Lond.) 266: 271-273.

Woodrow, G., and Schatz, G. 1979. The role of oxygen in the biosynthesis of cytochrome *c* oxidase of yeast mitochondria. J. Biol. Chem. 254: 6088-6093.

Wolf, K., and Del Giudice, L. 1988. The variable mitochondrial genome of ascomycetes: organization, mutational alterations, and expression. Adv. Genet. 25: 185-308.

Wolstenholme, D.R. 1992. Animal mitochondrial DNA: structure and evolution. Int. Rev. Cytol. 141: 173-216.

Woudt, L.P., Pastink, A., Kempers-Veenstra, A.E., Jansen, A.E.M., Mager, W.H., and Planta, R.J. 1983. The genes coding for histone *H3* and *H4* in *Neurospora crassa* are unique and contain intervening sequences. Nucleic Acids Res. 11: 5347-5360.

Wu, M., and Tzagoloff, A. 1989. Identification and characterization of a new gene *(CBP3)* required for the expression of a yeast coenzyme QH_2-cytochrome *c* reductase. J. Biol. Chem. 264: 11122-11130.

Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33: 103-119.

Yin, S., Heckman, J., and RajBhandary, U.L. 1981. Highly conserved GC rich palindromic DNA sequences which flank tRNA genes in *Neurospora crassa* mitochondria. Cell 26: 325-332.

Zennaro, E., Grimaldi, L., Baldacci, G., and Frontali, L. 1985. Mitochondrial transcription and processing of transcripts during release from glucose repression in "resting cells" of *Saccharomyces cerevisiae*. Eur. J. Biochem. 147: 191-196.

Appendix

Antibiotics added to bacterial media

Antibiotics	Final concentration (μg/ml)
ampicillin	100
tetracycline-HCl	20

LB Broth

10 g	bacto-tryptone (Difco)
5 g	yeast extract (Difco)
5 g	NaCl
1 g	D-glucose

The solution was made up to 1 litre with distilled water and sterilized by autoclaving for 20 minutes at 121°C. Solid medium for plates was made by adding agar (1.5%) prior to autoclaving. Top agar and top agarose were made by adding agar and agarose, repectively, to 0.7%.

D.M. Salts + Thiamine + Glucose

50 ml	20X D.M. stock
1 ml	10% MgSO4
1 ml	10 mM thiamine

The solution was made up to 500 ml with water and sterilized by autoclaving. A sterile 1% (w/v) solution of D-glucose (500 ml) was added when the above solution had cooled.

20X D.M. Stock

20 g	$(NH_4)_2SO_4$
60 g	KH_2PO_4
140 g	K_2HPO_4

This was made up to 1l with water and 5 ml of chloroform was added as a preservative.

Vogel's Medium

20 ml	50X Vogel's stock
1 ml	1X trace elements
1 ml	biotin solution
15 g	table sugar

Supplements (200 µg/ml of L-amino acids and 10 µg/ml of vitamins) were added when required. The solution was made up to 1l with water before autoclaving.

50X Vogel's

125 g	$Na_3citrate{\cdot}2H_2O$
250 g	KH_2PO_4
100 g	NH_4NO_3
10 g	$MgSO_4{\cdot}7H_2O$
5 g	$CaCl_2{\cdot}2H_2O$

The solution was made up to 1l with water. Chloroform (5 ml) was added as a preservative.

4X trace elements

50 g	$citric\ acid{\cdot}1H_2O$
50 g	$ZnSO_4{\cdot}7H_2O$
10 g	$Fe(NH_4)_2(SO_4){\cdot}6H_2O$
2.5 g	$CuSO_4{\cdot}5H_2O$
0.5 g	$MnSO_4{\cdot}1H_2O$
0.5 g	H_3BO_3
0.5 g	$Na_2MoO_4{\cdot}2H_2O$

20 mg	$CoCl_2$

Water was added to 1l. The 4X stock was diluted to prepare a 1X solution. Both solutions were stored at 4°C.

Biotin solution

Biotin (5 mg) was dissolved in a final volume of 400 ml of 50% (v/v) ethanol and stored at 4°C.

Viability plates

20 ml	50X Vogel's
1 ml	biotin solution
1 ml	1X trace elements
15 g	agar

Supplements were added as required (10 µg/ml of vitamins) and the solution was made up to 900 ml with water. After autoclaving, 100 ml of 10X sugars solution was added and plates were poured.

Benomyl transformation plates

Benomyl containing transformation plates consist of all of the same ingredients of viability plates, except they also contain benomyl. After autoclaving, 2 ml of benomyl solution (0.25 mg/ml in 95% ethanol) was added, and the plates were poured.

10X Sugars

200 g	L-sorbose
5 g	D-fructose
5 g	D-glucose
2 g	myo-inositol

Water was added to 1l and the solution was sterilized by autoclaving.

Top agar

20 ml	50X Vogel's stock
182 g	sorbitol
1 ml	1X trace elements

10 ml	vitamins mix
15 g	agar

Required supplements were added (10 µg/ml) and the solution was made up to 900 ml. The solution was autoclaved, and 100 ml of 10X sugars solution was added. For determining sphaeroplast viability, no benomyl was added. For transformations, 2 ml of benomyl soluton (0.25 mg/ml in 95% ethanol) was added after autoclaving.

Vitamins Mix

30 mg	riboflavin
100 mg	thiamine
75 mg	pyridoxine
5 ml	*p*-aminobenzoic acid (2mg/ml)
200 mg	choline chloride
1 ml	folic acid (5 mg/ml)
4 g	myo-inositol
0.5 g	adenine sulfate
0.5 g	uracil
0.5 g	L-methionine
0.5 g	L-arginine
1 g	L-lysine
0.1 g	L-threonine
0.5 g	L-serine
0.2 g	L-homoserine
0.5 g	L-histidine
0.1 g	L-isoleucine
0.2 g	L-valine
0.2 g	L-leucine
0.5 g	L-proline

Riboflavin was dissolved in 100 ml of water containing 4 drops of 5 N NaOH before adding to the rest of the solution. Water was added to 1 l, and the solution was filter sterilized. The solution was stored in a dark bottle at 4°C.

Guanidine solution

118.2 g	guanidium isothiocyanate
5 ml	1 M sodium acetate (pH 5.2)
3.85 mg	dithiothreitol (DTT)
1.25 g	N sarkosyl

Guanidine was dissolved in DEPC-treated, sterile distilled water and sodium acetate by heating at 65°C. Then DTT and sarkosyl were added. The pH was adjusted to about 5.5 and more water was added to a volume of 250 ml. The solution was filter sterilized and stored at room temperature.

poly A loading buffer

0.5 M LiCl
10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.1% SDS

Middle wash buffer

0.15 M LiCl
10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.1% SDS

Elution buffer

10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.05% SDS

50X Denhardt's

1% (w/v) Ficoll (400 000 MW)
1% (w/v) polyvinypyrrolidone (360 000 MW)
1% (w/v) Bovine Serum Albumin

The solution was filter sterilized and stored at -20°C in 25 ml aliquots.

20X SSC

3 M sodium chloride
0.3 M sodium citrate, pH7

Oligolabelling buffer

Prepared by mixing 3 different solutions, A, B and C, in a ratio of 20 : 50 : 30.

Solution A: 1 ml 1.25 M Tris-Cl pH 8, 0.125 M $MgCl_2$
18 µl β-mercaptoethanol
5 µl 100 mM dATP, 3 mM Tris-Cl pH 7, 0.2 mM EDTA
5 µl 100 mM dTTP, 3 mM Tris-Cl pH 7, 0.2 mM EDTA
5 µl 100 mM dGTP, 3 mM Tris-Cl pH 7, 0.2 mM EDTA

Solution B: 2 M HEPES, pH 6.6

Solution C: Hexadeoxyribonucleotides made to 90 OD units/ml in 3 mM Tris-HCl, pH 7.0, 2 mM EDTA.

SM buffer

5.8 g NaCl
2.0 g $MgSO_4 \cdot 2H_2O$
50 ml 1 M Tris-Cl, pH 7.5
5 ml 2% gelatin
Add distilled water to 1 l and autoclave.

2X YT medium

10 g yeast extract (Difco)
16 g bacto-tryptone (Difco)
10 g NaCl
Add distilled water to 1 l and autoclave.

Sequencing termination mixes

Regular mixes

ddG Termination mix: 80 µM of each of dGTP, dATP, dCTP and dTTP
8 µM ddGTP, 50 mM NaCl

ddA Termination mix: 80 µM of each of dGTP, dATP, dCTP and dTTP
8 µM ddATP, 50 mM NaCl

ddC Termination mix: 80 µM of each of dGTP, dATP, dCTP and dTTP

8 μM ddCTP, 50 mM NaCl

ddT Termination mix: 80 μM of each of dGTP, dATP, dCTP and dTTP
8 μM ddTTP, 50 mM NaCl

dITP mixes

ddG Termination mix: 160 μM dITP, 80 μM of each of dATP, dCTP, dTTP
1.6 μM ddGTP, 50 mM NaCl

ddA Termination mix: 80 μM of each of dITP, dATP, dCTP and dTTP
8 μM ddATP, 50 mM NaCl

ddC Termination mix: 80 μM of each of dITP, dATP, dCTP and dTTP
8 μM ddCTP, 50 mM NaCl

ddT Termination mix: 80 μM of each of dITP, dATP, dCTP and dTTP
8 μM ddTTP, 50 mM NaCl

Polyacrylamide sequencing gels (6%)

24 ml	40% (w/v) acrylamide/bisacrylamide (19/1)
80 g	urea
16 ml	1 M Tris-borate, pH 8.3, 20 mM EDTA
60 ml	distilled water

The urea was dissolved by placing the solution in a 50-60°C water bath and the solution was filtered. For each short gel (40 cm x 20 cm x 0.3 mm), 35 ml of the solution was used, whereas 70-80 ml of solution was used for long gels (80 cm x 20 cm x 0.3 mm). Prior to pouring short gels, 10 μl of N, N, N', N' tetramethyl ethylenediamine and 250 μl of 10% ammonium persulfate were added (per gel) to polymerize each gel. Twice as much of each reagent was added to each long gel.

Protein gels

Stacking gel

(3.75% acrylamide, 0.5% SDS)

2.5 ml	30% acrylamide/bisacrylamide (29/1) (w/w)
5 ml	0.5 M Tris-HCl, pH 6.8

1 ml 10% SDS (filtered)
11.5 ml distilled water

Resolving gel

(15% acrylamide, 1% SDS)

75 ml 30% acrylamide/bisacrylamide (29/1) (w/w)
19 ml 3 M Tris-HCl, pH 8.8
15 ml 10% SDS (filtered)
41 ml distilled water